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# *TRICHOMONAS MURIS*, PARASITIC IN THE OXYURID NEMATODES, *ASPICULARIS TETRAPTERA* AND *SYPHACIA OBVELATA*, FROM WHITE MICE

BY HANS THEILER AND SEYMOUR M. FARBER

*From the Department of Comparative Pathology, Harvard University Medical School and School of Public Health, Boston, Massachusetts*

(With 1 Figure in the Text)

IN the course of a study of the parasites of white mice, it was observed (S. M. F.) that certain of the nematodes in the large intestine harboured actively motile flagellates. Further search soon revealed that this was not a rare occurrence and it was decided to study the phenomenon. It soon became evident that *Aspicularis tetraptera* may frequently be infected with *Trichomonas muris*. Later it was found that *Syphacia obvelata*, which inhabits the caecum of the mouse, may be similarly infected. A preliminary report of this work was published (1932).

Kudo & Hetherington (1922) described a microsporidian parasite of the spirurid nematode, *Protospirura muris*, of the common house mouse, and discussed earlier reports of parasites of the cat ascarid *Toxocara mystax*. According to Graybill & Smith (1920) lowered resistance of the host by infection with *Heterakis gallinae* might favour the organism causing blackhead in turkeys. Smith & Graybill (1920) also showed that blackhead may be produced in chickens by feeding to the birds embryonated eggs of the heterakid. These findings received ample confirmation in the hands of Tyzzer & Fabyan (1920), and Tyzzer (1926, 1934) conclusively proved that *Histomonas meleagridis* is transmitted by the heterakid and that the birds acquire the disease through the ingestion of the embryonated eggs of the helminth. He was able to demonstrate the protozoön in the alimentary tract of larvae obtained from chicks which had been fed with embryonated eggs, derived from gravid females taken from the caeca of birds which had recovered from blackhead and which had become carriers of *H. meleagridis*. He was, however, not able to demonstrate the organisms microscopically in the reproductive organs of the female worms or in the eggs. Thomson (1925) reported infections of *Giardia viscaciae* occurring in the lumen of the intestine of a nematode (*Viannella* sp.) found in the small intestine of a South American rodent (*Viscacia viscacia*). No free trophozoites or cysts of *Giardia viscaciae* were found in the intestines or intestinal contents of the two dead hosts examined. Another reported occurrence of protozoan infection in a parasitic nematode is furnished by Graham (1935), who found *Giardia* in the intestine of *Cooperia oncophora* recovered from a young bull.

### Material and methods

The mice used were all from the same stock and received approximately the same handling and treatment before they became subjects for this investigation. All were members of successive groups of animals from the same breeder and were survivors from protection experiments. As a routine procedure the mice were killed and the caecum and large intestine removed and opened. The degree of infection with trichomonads in the caecum and in the large intestine was noted, the nematodes *Aspicularis tetraptera* and *Syphacia obvelata* were collected and counted and the worms examined for the presence of flagellates in the chyle-intestine. Though particular attention was paid only to the trichomonad and nematode infections, the occurrence of other parasites of the mice

Table I. *Distribution of* Trichomonas muris *and* Aspicularis tetraptera *infections*

| | | | | *T. muris* infections in mice | | *A. tetraptera* infections in mice | | *T. muris* infections in worms | |
|---|---|---|---|---|---|---|---|---|---|
| Group No. | Experimental treatment | No. of mice | Not recorded | Negative | Positive | Negative | Positive | Positive | Negative |
| 1 | Stock | 24 | 24 | — | — | 5 | 19 | 11 | 8 |
| 2 | Stock | | — | 12 | — | 5 | 7 | 1 | 6 |
| | | | — | — | 15 | 2 | 13 | 9 | 4 |
| | | 27 | — | 12 | 15 | 7 | 20 | 10 | 10 |
| 3 | Controls | | — | 19 | — | 2 | 17 | 1 | 16 |
| | | | — | — | 6 | 2 | 4 | 2 | 2 |
| | | 25 | — | 19 | 6 | 4 | 21 | 3 | 18 |
| 4 | Fed *T. muris* | | — | 13 | — | 2 | 11 | 0 | 11 |
| | | | — | — | 12 | 3 | 9 | 3 | 6 |
| | | 25 | — | 13 | 12 | 5 | 20 | 3 | 17 |
| 5 | Fed *A. tetraptera* | | — | 13 | — | 2 | 11 | 1 | 10 |
| | | | — | — | 12 | 2 | 10 | 5 | 5 |
| | | 25 | — | 13 | 12 | 4 | 21 | 6 | 15 |
| 6 | Fed *T. muris* and *A. tetraptera* | | — | 9 | — | 2 | 7 | 0 | 7 |
| | | | — | — | 4 | 0 | 4 | 2 | 2 |
| | | 13 | — | 9 | 4 | 2 | 11 | 2 | 9 |
| | | 139 | 24 | 66 | 49 | 27 | 112 | 35 | 77 |

was recorded. For convenience the findings were tabulated and are presented in summarised form in Tables I and II. In some cases, in which the helminth infections were unusually heavy, a certain number of worms were examined and the total number present estimated. In the tabulation and analysis of the findings, however, only the data for the worms actually examined are utilised.

Six groups of mice were studied (see Table I). In the preliminary group (No. 1) no record of the presence or absence of *Trichomonas muris* in the mice is available. Group 2 represents a random selection of mice from the general stock. The remaining four were experimental groups and were treated as follows: group 4 was fed with *T. muris*, group 5 was fed with embryonated eggs of *Aspicularis tetraptera*, group 6 was fed with *Trichomonas muris* and embryonated eggs of *Aspicularis tetraptera*, and group 3 served as controls for groups 4, 5 and 6. Each of the four experimental groups consisted of twenty-five animals

at the beginning of the experiment, but in the case of group 6, after thirteen of the mice had been examined, the remainder died and were discarded without examination.

A large number of fixed and stained preparations were made for a comparative study of the morphology of the flagellates in the helminth and those free in the lumen of the intestine of the mice. Measurements were made of trichomonads from the chyle-intestine of *A. tetraptera* and also of the organisms free in the intestine of the mice. In addition, serial sections of infected and uninfected worms were studied to determine whether there were injuries to any organs or tissues of the helminth. At the same time a careful search was made of all the nematode tissues in an effort to find whether the flagellates are able to invade organs, tissues or cells. The fixatives used for the worm and faecal smears included Schaudinn's, Flemming's, Zenker's and Champy's and most of the preparations were stained by the method of Heidenhain. The worms for *in toto* mounts and for serial section were fixed in hot alcohol and the sections stained with iron-haematoxylin.

Table II. Trichomonas muris *infections in* Aspicularis tetraptera *from* Trichomonas*-positive mice*

| | | | | | *T. muris* infections in mice | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *A. tetraptera* infections in *Trichomonas*-positive mice | | | | | | |
| | | | | | | | *T. muris* infections in *A. tetraptera* from *Trichomonas*-positive mice | | | | |
| Group No. | Experimental treatment | No. of mice | No. of mice infected | % | No. of mice | % | No. of mice | % | Worms examined | Worms infected | % |
| 2 | Stock | 27 | 15 | 55·6 | 13 | 86·7 | 9 | 69·2 | 57 | 38 | 66·7 |
| 3 | Control | 25 | 6 | 24·0 | 4 | 66·7 | 2 | 50·0 | 61 | 12 | 19·7 |
| 4 | Fed *T. muris* | 25 | 12 | 48·0 | 9 | 75·0 | 3 | 33·3 | 62 | 27 | 43·5 |
| 5 | Fed *A. tetraptera* | 25 | 12 | 48·0 | 10 | 83·3 | 5 | 50·0 | 108 | 45 | 41·7 |
| 6 | Fed *T. muris* and *A. tetraptera* | 13 | 4 | 30·8 | 4 | 100·0 | 2 | 50·0 | 17 | 12 | 70·7 |
| | | 115 | 49 | 42·6 | 40 | 81·6 | 21 | 52·5 | 305 | 134 | 43·9 |

In addition twenty-four mice in group 1, in which the presence or absence of *Trichomonas* infections was not recorded, yielded seventy-one infected worms (23·4 per cent.) out of 366 *Aspicularis tetraptera* examined. Also three *Trichomonas*-negative mice (one each) from groups 2, 3 and 5 (see Table I) yielded twenty-three worms, six of which (25·1 per cent.) were infected with *Trichomonas*. Thus a total of 211 *Trichomonas*-infected *Aspicularis tetraptera* (30·4 per cent.) were encountered in 694 worms obtained from 112 mice out of 139 examined.

## OBSERVATIONS

Infections with *Aspicularis tetraptera* were encountered in 112 out of 139 mice examined. Infections with *Trichomonas muris* were found to occur in forty-nine out of 115 of the same mice (there being no record of the presence

or absence of infection in the remaining twenty-four). The distribution of the worm and protozoan infections in the mice are given in Table I, in which the occurrence and distribution of trichomonad infections in the worms are also recorded. Worms infected with the flagellate were recovered from thirty-five mice. Infections with *T. muris* were recorded as occurring in twenty-one of these mice: three mice (one each from groups 2, 3 and 5 (Table I)) were reported uninfected, and no record of protozoan infections was made for the remaining eleven animals. Forty of the forty-nine mice infected with the trichomonad also harboured *Aspicularis tetraptera*, but only twenty-one of these mice yielded worms containing the flagellate. Twenty-two of the 115 mice, in which complete examinations were made, were free of both infections.

An analysis of the findings pertaining to the 115 mice (see Table II) for which the data were complete reveals the fact that forty-nine (42·6 per cent.) of the mice were infected with *Trichomonas muris*, of which forty (81·6 per cent.) also harboured *Aspicularis tetraptera*. Twenty-one of the forty (52·5 per cent.) flagellate and worm-infected mice contained worms harbouring the trichomonad and yielded 305 *Aspicularis tetraptera*, 134 (44 per cent.) of which were infected with *Trichomonas muris*. Considerable variation in the degree of infection was found to occur in the several groups of animals examined. Flagellate infections in lots of worms from different groups of mice varied from 19·7 to 70·7 per cent. Similarly there was a variation of from 33·3 to 69·2 per cent. of worm incidence in mice infected with trichomonads, while the incidence of the flagellate infections in the mice varied from 24 to 55·6 per cent. Taking into consideration the worms collected from the twenty-four mice in group 1 (see Table I) for which the data are incomplete and adding the worms collected from the three trichomonad-negative mice, it was found that 112 (80·6 per cent.) out of 139 mice yielded 694 worms, of which 211 (30·4 per cent.) were infected with the flagellate. In reality, however, the 211 infected worms were collected from thirty-five mice (25·2 per cent.) out of the 139 examined (see Table I).

Some of the infected *Aspicularis tetraptera* individuals contained only one or few trichomonads, but in the great majority of individuals *Trichomonas muris* was present in moderate numbers, and in a few cases the chyle-intestine was greatly distended and literally packed with the flagellates. Male worms were found to be infected as frequently as females. Older larvae were found to harbour infections, but no trichomonads or other protozoa were found in young larvae. In the lighter and moderately heavy infections peristaltic waves could be seen moving alternately backward and forward along the length of the chyle-intestine. There was invariably a rhythmic contraction of the valvular apparatus in the oesophageal bulb, and on a number of occasions material was observed to pass from the oesophagus into the chyle-intestine. The valve mechanism seems to control perfectly the passage of material in one direction, and only once has material been observed to pass from the chyle-intestine into the oesophagus. This occurred in a worm which was so greatly distended that contraction of the intestine was no longer possible. The contraction of the

valvular apparatus is followed by a wave of contraction passing downward to the cloaca or anus of the male and female respectively. The downward wave is followed immediately by a wave in the opposite direction. The contractions succeed each other at relatively short intervals and may continue for an indefinite period of time. The contents of the intestine pass up and down and are thoroughly churned in the process, and the flagellates have repeatedly been observed moving from end to end of the intestine, without apparently suffering any ill-effects. The rate of translation of the flagellates from end to end of the chyle-intestine is many times greater than the rate of progression attributable to any active movement of the trichomonads, which are carried passively as a result of the physiologic mechanism of the helminth. Though this mechanism has been watched for considerable periods of time on numerous occasions, the passage of material from the intestine through the cloaca or anus has never been observed and it is not known whether the trichomonads escape into the surrounding medium or whether they are destroyed and digested. Dividing forms have been recognised in the chyle-intestine of the living worm and have also been studied in stained coverslip preparations.

Occasionally worms are seen at rest, with no movement of the valvular apparatus or of the chyle-intestine taking place. Such individuals, when infected, show a considerable degree of dilation of the anterior portion of the chyle-intestine immediately contiguous to the oesophageal bulb. The flagellates may be recognised readily by the characteristic and regular movements of the undulating membrane and the type of progression which results therefrom. Under suitable lighting conditions the movement of the undulating membrane may easily be studied, but it has not been possible to follow accurately the type of motion undergone by the anterior flagella. That it is of a lashing nature is clearly discernible, but it has not been possible to determine whether the beat in one direction is faster or slower than that in the opposite direction. The axostyle may also be readily recognised in organisms moving freely in the intestine of the worm.

The infection in the worms has been studied in living material, in stained sections and in preparations mounted *in toto*. The flagellate itself was studied in smears made from worms crushed on cover-slips and in faecal smears. A careful search was made of all tissues and organs of the worms. The most constant finding was some distention of the chyle-intestine, but in no case was any evidence obtained of any injury to the worms. The trichomonads were never found intracellularly and there was no evidence that the organisms are able to leave the lumen of the intestine to penetrate the epithelium or to invade the tissues surrounding the intestine. Whenever the protozoa were found they were invariably restricted to the intestinal tract. In view of Tyzzer's demonstration of the passage of *Histomonas meleagridis* through the egg of the oxyurid worm *Heterakis gallinae* and the production of blackhead in susceptible chicks and turkeys by feeding with embryonated eggs, particular attention was paid to the reproductive organs. Tyzzer (1926, 1934) was able to demonstrate

*Histomonas meleagridis* in the intestinal tract of larvae of *Heterakis gallinae*, obtained by feeding with eggs embryonated in 1·5 per cent. nitric acid, but has not been able to demonstrate microscopically the organisms in the reproductive organs of mature worms, or in eggs the feeding of which, after embryonation, produces blackhead. Though *Histomonas meleagridis* was demonstrated in the alimentary tract of the larvae it was never as numerous as *Trichomonas muris* in the intestine of *Aspicularis tetraptera*. The finding of flagellates in the chyle-intestine of *A. tetraptera* in *Trichomonas*-free mice suggested that egg transmission may occur also in this case. It seemed probable that such a phenomenon might more readily be demonstrable as heavy infections with *T. muris* are the rule rather than the exception in *Aspicularis tetraptera*. As already pointed out above, trichomonads were never encountered in any position other than the lumen of the chyle-intestine, nor has their occurrence been observed in young larvae. Becker (1933), however, has observed living *Trichomonas muris* in eggs of *Syphacia obvelata*.

Our stock of mice was found to be infected with *Entamoeba muris*, *Giardia muris* and *Eimeria falciformis* and in a very limited number of cases with *Chilomastix bettencourti*. At infrequent intervals a few individuals of *Aspicularis tetraptera* were found to be harbouring *Entamoeba muris* or *Giardia muris* in small numbers and one on occasion one worm containing a single specimen of *Chilomastix bettencourti*. On several occasions *Syphacia obvelata* was found to be infected with *Trichomonas muris*, but such infections did not occur as frequently nor were they as heavy as the trichomonad infections in *Aspicularis tetraptera*.

That the trichomonads in their turn may be parasitised is evident from the fact that *Sphaerita* was encountered quite frequently in them. On several occasions *Trichomonas muris* in the chyle-intestine of *Aspicularis tetraptera* was found to be infected with *Sphaerita*, but less frequently than *Trichomonas* living free in the lumen of the alimentary tract of the mice. These infections in the flagellates were recognised only in fixed and stained preparations.

### Morphology and size

As seen in our material the morphology agrees closely with descriptions of *Trichomonas muris* furnished by various authors. The appearance of the various organelles and structures varies considerably with the fixatives and staining methods used. The following structures have been recognised: cytostome, undulating membrane, nucleus and centriole (*sensu* Cleveland, 1934) with the various organelles arising from it, *i.e.* anterior flagella, marginal axoneme of the undulating membrane with its free trailing terminal portion, chromatic basal rod, parabasal and axostyle. From observations made on dividing individuals (p. 157) it is evident that the structures previously described as "blepharoplast" and "rhizoplast" by all authors who have recognised them should be considered as a single unit, and it is from this

body that the division of the nucleus and the growth of the new organelles is initiated. Thus, as Cleveland has shown, this structure can only be regarded as the centriole. In the interphase it consists of a single granule with a fibre running towards the nucleus. Contact may or may not be made with the nuclear membrane and this "rhizoplast" may be extremely difficult to demonstrate in some preparations. We have, however, seen it frequently enough to be satisfied that it is a process of the centriole in the sense described by Cleveland. Several authors have described the "blepharoplast" or proximal portion of the centriole as being made up of two or more granules, which may or may not be embedded in a matrix. In an examination of exceedingly rich material it has not been possible, except in extremely rare instances, to resolve this structure. In one or two cases two granules lying side by side have been recognised, but more frequently a crescentic structure has been encountered, which consists probably of a band connecting the two granules constituting the proximal portions of the old and new centrioles respectively.

The cytostome is anteriorly located and ventral to the anterior flagella which are three in number and have a common origin with the axostyle, parabasal, chromatic basal rod and axoneme of the trailing flagellum. The cytostome is a simple indentation of the pellicle of the organism and not supported by fibres of any description. The axostyle is a clear hyaline structure arising as a fibre from the proximal portion of the centriole, but soon widens out into a broad band running posteriorly and ventral to the nucleus tapering to a point, often with a terminal fibril, after piercing the body wall. At the point where the axostyle protrudes through the pellicle of the animal there is a condensation of material forming more or less of a collar. In *T. muris* we have never observed intra-axostylar granules but have often seen a row of a variable number of granules in close proximity with and dorsal to the axostyle. This row of granules is frequently demonstrable and closely resembles the row found almost constantly ventral to the chromatic basal rod. The axostyle is a flexible and rather elastic structure and in fixed specimens may assume extremely varied positions but all bending which it may undergo always results in the formation of smooth curves. This structure is not capable of active movement but may be passively bent in all directions as a result of cytoplasmic or other contractions. The parabasal arises from the centriole as a fibril and widens into a sausage-shaped structure one-third to one-half the length of the axostyle and runs posteriorly and dorsal to the nucleus. The parabasal is only seen after the use of certain fixatives, never stains very deeply and is easily destained as Wenrich (1921) and others have already noted.

The chromatic basal rod, or costa of some authors, is the most dorsal structure. It is relatively rigid and supports the undulating membrane which is a fold of the pellicle extending one-half to three-quarters the length of the organism. The chromatic basal rod arises also from the centriole, widens rapidly to its maximum width and then tapers off gradually to a fine point at its distal extremity. The undulating membrane varies considerably in width

from individual to individual, is freely movable and carries in its margin the axoneme of the flagellum which terminates posteriorly in a free trailing portion. This flagellum as well as the three anterior free flagella also arise from the proximal anterior granule of the centriole. The anterior flagella are often as long or longer than the body of the animal but may be shorter and are always very actively moving in the living animal, even during division. The flagella may be moved independently of each other but are more often to be seen lashing together in unison. Such movements though regularly rhythmic are, however, exceedingly difficult to follow owing to the great activity of the animal. The nucleus, situated in the anterior portion of the organism and lying between the axostyle and parabasal, is usually ovoid or subspherical. In the interphase or resting stage it shows fine granular particles of chromatin regularly distributed throughout and enclosed in a delicate nuclear membrane which persists during the division process.

The size of *T. muris* varies considerably in fixed and stained preparations and is to some extent dependent on the fixative used. This observation has been recorded by Wenrich and others. In our material the organism was found to vary from 6·7 to 24 $\mu$ in length by 4·7 to 18 $\mu$ in width. In preparations made from faecal material fixed in Champy's fluid and stained with Heidenhain's iron-haematoxylin measurements made on 82 individuals gave an average of 13·7 $\mu$ in length by 7·9 $\mu$ in width. One hundred and forty-five individuals similarly stained after fixation in Schaudinn's fluid with acetic acid yielded average dimensions of 12·8 by 8·1 $\mu$. For 105 individuals after fixation in Zenker's and the same stain the average dimensions were 14·1 by 10·1 $\mu$. The mean dimensions of 104 individuals from *Aspicularis tetraptera*, fixed in Schaudinn's fluid without acetic and stained by the Heidenhain method, were 13·8 by 9·3 $\mu$. Similar material similarly stained after fixation with Champy's fixative gave an average of 13·8 by 10·4 $\mu$ for 119 individuals. There is thus some slight difference in size attributable to the fixative used, but for purposes of charting the results these differences have been disregarded. All the measurements for organisms in preparations made from faecal material have been grouped together regardless of the fixative used and a frequency curve plotted (broken line, Fig. 1). There are three apparent peaks in this curve, which are, however, to be considered as showing the influence of the three fixatives used and not as indicating the occurrence of three size-races or of three distinct species of organisms. A similar curve embodying the measurements of trichomonads in the chyle-intestine of *A. tetraptera* shows that the size distribution of the organisms closely follows that of the trichomonads in the faecal material in the large intestine from which the oxyurids were recovered. This material was fixed in two different fixatives and this is reflected in the pronounced shoulder in what is otherwise a strictly unimodal curve (solid line, Fig. 1). When similar curves are plotted for the measurements of the widths of the trichomonads from the two separate sources an even closer correlation in size distribution is indicated. There is thus no doubt that the organisms in the chyle-intestine of the helminth

are of the same species as the trichomonads found free in the large intestine of the mice from which the worms were recovered.

## DIVISION

Except for some differences in interpretation our observations, in the main, confirm Wenrich's description (1921) of division in *Trichomonas muris*. We will not at this time give a detailed and illustrated account of division, but merely a short résumé of the interpretation of the phenomena, stressing those points in which our view differs from that of Wenrich. The points not specifically discussed may be assumed to agree with Wenrich's findings. The earlier descriptions of mitosis in trichomonads (Wenyon, 1907; Dobell, 1909; Martin & Robertson, 1911; Kuczynski, 1914, 1918; and Kofoid & Swezy, 1915), have already been adequately discussed by Wenrich.

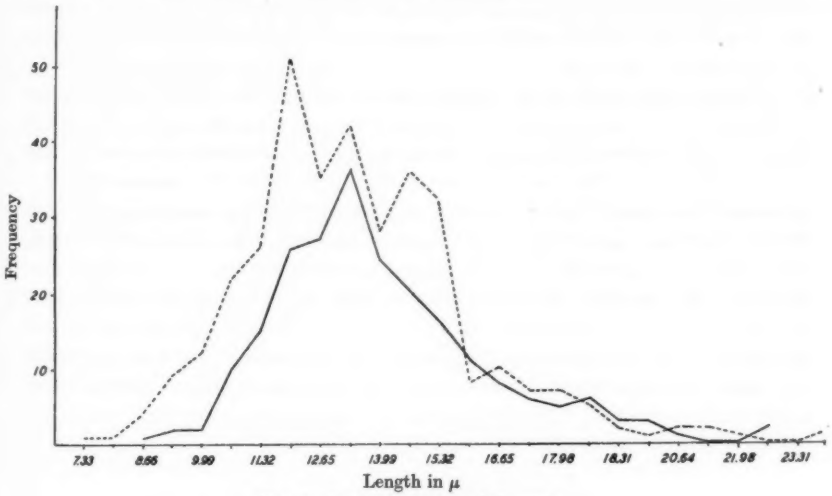


Fig. 1. Size distribution of *T. muris*.
Solid line—length of *T. muris* from *A. tetraptera*.
Broken line—length of *T. muris* from lumen of intestine of mouse.

The first sign of division is almost invariably seen in the "blepharoplast". As already noted above, Cleveland (1934) has shown that this organ is but a part of the mechanism initiating and controlling the division process and that the "blepharoplast" and "rhizoplast" together constitute the centriole. The order in which the changes occur during division forms an orderly sequence rather than a series of unrelated events occurring haphazardly. The first change is the transformation of the "blepharoplast" or proximal portion of the centriole into a crescentic body, which we interpret to consist of two granules (or possibly aggregations of centriolar substance) joined together by a relatively broad band. As these draw a little further apart a new chromatic basal rod begins to form

and new anterior flagella soon appear. Concurrently with this train of events and after the bond with the old centriole has been severed, there is a growth of the new centriole in the direction of the nucleus. This continues until contact with the nuclear membrane is established and formation of the central spindle then occurs. Not infrequently it happens that one or even two of the old flagella may be carried with the new centriole. In such cases each of the daughter centrioles make up the full complement of flagella. We have not been able to recognise any marked changes in the nucleus during the early part of the centriolar portion of the prophase. Nuclear changes have not been clearly recognised till late in the prophase or early metaphase and we have only in exceptionally rare instances been able to count the chromosomes, which we have found to be six in number. We have, however, never seen them as clearly as depicted in Wenrich's illustrations. While these nuclear changes have been taking place the new marginal flagellum and the new undulating membrane have made their appearance and have grown out considerably from their point of origin in the centriole.

With the formation of the central spindle and the movement of the poles of the spindle to opposite sides of the nuclear membrane the nucleus becomes elongate and rotates to lie approximately at right angles to the long axis of the animal. At about this time the axostyle shows signs of degeneration and gradually disappears. The new axostyles arising from the proximal part of each of the centrioles make their appearance at the anaphase or possibly during early telophase, when the new parabasal suddenly appears as an outgrowth from the new centriole. We have not been able to recognise any stages in the growth of the new parabasal but have found this structure fully formed before the division of the nucleus is completed. The full complement of organelles including the new cytostome may thus be duplicated and complete before division of the nuclear membrane occurs. We have no evidence as to the manner in which the new cytostome is formed.

## Discussion

The observations recorded above, as well as those reported by the various authors cited, indicate that a number of protozoa are capable of living in the intestine of parasitic worms of vertebrate hosts. In the present study it has been found that the trichomonads may be present in the intestine of the nematode even though it was not possible to demonstrate the protozoön in the caecum and small intestine of the mice that harboured the helminths. The finding of dividing flagellates in the chyle-intestine of the oxyurid is further evidence that the protozoön is able to persist and maintain itself in this seemingly foreign habitat. Despite our failure to demonstrate flagellates in larvae, Becker's demonstration of living trichomonads in the ovum of *Syphacia obvelata* suggests the possibility, that the trichomonad may, under certain conditions, be transmitted from mouse to mouse as a by-product of the worm infection. That a protozoan infection may be regularly transmitted through

the agency of an helminth has been conclusively proved by Tyzzer (1926, 1934) and it seems improbable that this is an isolated and unique phenomenon, even without any direct experimental evidence of other protozoan infections transmitted in this manner.

Although all the protozoa present in the intestine of mice with the exception of the coccidium, were found in the intestine of the oxyurids, such findings were relatively infrequent as compared with the trichomonad infections in the worms. It was also characteristic of these infections that the protozoa occurred only in small numbers and never filled the intestine of the worms, as was not infrequently the case in the trichomonad infections. Thus it would appear that the trichomonad is better able to adapt itself to this strange new environment than are the other protozoa. We have seen that *Trichomonas muris* may undergo division in the intestine of the worms, but no such evidence presented itself with regard to the other protozoa. This, however, may be of no significance as comparable numbers of these organisms were not examined.

Neither Thomson (1925) nor Graham (1935) were able to find *Giardia* in the intestines or intestinal contents of their host animals. Thomson's material was obtained from dead animals and Graham's material was not examined until nine hours after the host was slaughtered. In our study all examinations were made immediately after the mice were killed and the failure to demonstrate *Trichomonas muris* in some of the mice harbouring the trichomonad-infected oxyurids is probably of greater significance than the parallel observations reported by Thomson and by Graham, for depending on the conditions under which material is stored flagellates may disappear in a comparatively short time from faecal material. It was not possible to determine whether the *Trichomonas*-free mice from which the *Trichomonas*-infected worms were recovered had had infections with the flagellate before infection of the worms had taken place.

## SUMMARY

*Trichomonas muris* infections were encountered in the oxyurid nematodes *Aspicularis tetraptera* and *Syphacia obvelata* parasitic in laboratory white mice. More rarely infections with other protozoa were encountered in the helminths. The distribution of both protozoan and helminth infections was studied, and the incidence of infected helminths determined. Dividing forms of *Trichomonas muris* occurred in the intestine of the worm as well as free in the lumen of the intestine of the mouse. An interpretation of certain findings in dividing forms is offered.

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# PREPARASITIC MOULTS IN *NIPPOSTRONGYLUS MURIS*, WITH REMARKS ON THE STRUCTURE OF THE CUTICULA OF TRICHOSTRONGYLES

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(With 13 Figures in the Text)

## Introduction

The life history and development of *Nippostrongylus muris* (syn. *Heligmosomum muris* Yokogawa 1920) were described by Yokogawa in 1922. Yokogawa reported only three complete moults in the course of the development of this nematode, one moult during the free-living stages and two moults during development in the host, and, although nematodes customarily undergo four moults, his observations have apparently been generally accepted.

Knowing that there are four moults as a rule among nematodes, Yokogawa attempted to reconcile his observations on the moulting of *Nippostrongylus muris* with the generally accepted facts of nematode development as usually observed. While it is improbable that it was Yokogawa's intention to offer two distinct theories in explanation of the apparent anomaly in the number of moults in *Nippostrongylus*, his paper actually contains two views concerning the stages in the parasitic development of the species; these views seem to the writer to be mutually inconsistent and exclusive.

According to one of Yokogawa's interpretations, the early development of the larvae in the lungs of rats involves a transition from what he regarded as the second (infective) to the third larval stage, an intervening moult being suppressed. Schwartz & Alicata, who in 1934 followed Yokogawa's view in this respect, have recently (1935) rejected this interpretation. These investigators (1935) state that although the infective larvae of *N. muris* are, as stated by Yokogawa, second-stage larvae as established by visible moults, the larvae in question must be regarded as homologous to the third-stage infective larvae of other trichostrongyles on the basis of their structure and behaviour. With regard to *Nippostrongylus* and the closely related genus *Longistriata*, investigated by Schwartz & Alicata, these writers believe that the first preparasitic moult is suppressed and that the single preparasitic moult corresponds to the second moult of other strongyles. They further express the opinion that the suppression of the first moult may be found to be a feature in the larval development of other genera of Heligmosomidae.

The second interpretation given by Yokogawa, regarding the develop-

mental stages of *Nippostrongylus muris*, is contained in his statement that the adults of this species possess two cuticulae, a condition which he considers indicative of the occurrence of an "incomplete fourth" moult. Although this view has apparently been ignored by other workers, it requires consideration because the three complete moults actually observed by Yokogawa plus the additional suppressed moult postulated by later investigators, fully account for the four moults and five stages characteristic of the development of nematodes. In connection with this point there is included in this paper a comparative study of the cuticle of the adults of *N. muris* and other trichostrongyles, with observations on the preparasitic development.

## Reinvestigation of the development and morphology of the preparasitic larvae of *Nippostrongylus muris*

Eggs removed from female *N. muris*, or isolated by means of the usual centrifugation and salt flotation technique from freshly passed faeces of albino rats experimentally infected with these parasites, were cultured in water at room temperature (20–26° C.) in small covered glass dishes. Larvae newly hatched from the eggs, as well as larvae more advanced in development, were killed, following removal from the culture, usually by means of a drop of iodine solution, to permit study of their structure. Living larvae were also studied in water or by means of a technique, recently described by Krull (1934), involving the use of egg albumen.

Newly hatched rhabditiform larvae, which were about 270–290$\mu$ long, had a very thin, transparent cuticle with lateral alae, but without resolvable striation. In larvae examined a few hours after hatching, the cuticle was separated slightly from the body in the post-anal region and extended slightly beyond the posterior tip of the long, fine tail (Fig. 1). This indication of the approach of an ecdysis appeared, therefore, unusually early in the life of the larvae. Larvae examined about 24 hours after they had hatched in water showed that further progress in the separation of the fine cuticle had occurred, and that a much thicker underlying new cuticle with prominent transverse striae had formed or was in process of formation, the new cuticle being particularly evident in the anal region and near the anterior extremity (Fig. 2). Larvae showing this condition were about 300–475$\mu$ long. It is evident that the larvae show the

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**Legends to figs. 1–6.**

*Nippostrongylus muris.*

Fig. 1. First-stage larva (posterior end) early in first ecdysis.

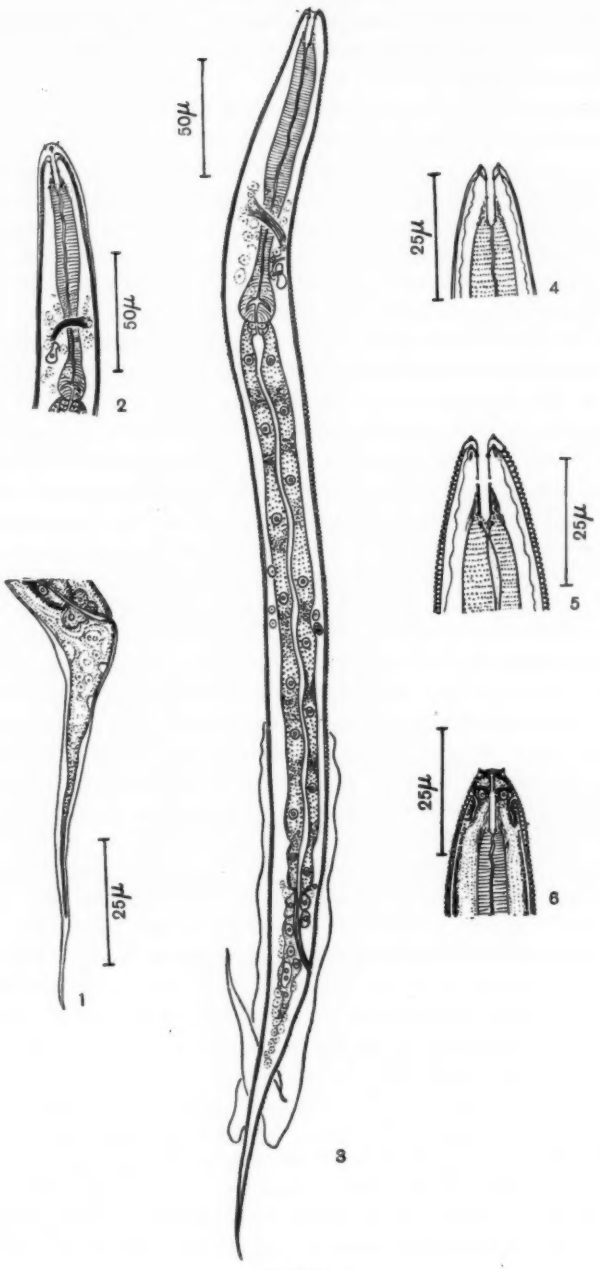
Fig. 2. First-stage larva (anterior end) in first ecdysis, showing newly formed underlying striated cuticle.

Fig. 3. Second-stage larva, shortly after the first moult, with the first-stage cuticle still adhering.

Fig. 4. First-stage larva (anterior extremity), showing morphology of buccal capsule.

Fig. 5. Second-stage larva (anterior extremity), showing morphology of buccal capsule.

Fig. 6. Third-stage larva (anterior extremity).




Figs. 1–6.

beginnings of an approaching ecdysis before they show any significant evidence of growth, a condition not previously reported in connection with life-history studies of trichostrongyles.

Larvae in more advanced phases of development were obtained from cultures consisting of faeces of experimentally infected rats and moist charcoal. Portions of the cultures were placed in a small Baermann apparatus at various intervals following their preparation. The first observations were on larvae from cultures prepared from an overnight accumulation of faeces from rats which had been confined in a clean cage. All of these cultures were later examined carefully for free-living nematodes with negative results.

Among the numerous larvae obtained in this manner from cultures prepared about 36–48 hours previously, a few were found in the act of casting off the first cuticle (Fig. 3). Five rhabditiform larvae, to which the partially cast off cuticle was still attached in the tail region, were 480, 530, 540, 580, and 590$\mu$ long, respectively. Larvae recovered from the cultures during the interval mentioned were by no means uniform in size and stage of development. Many were already in the second stage, but some first-stage larvae of various sizes were usually present. This accounts for the fact that the proportion of larvae undergoing the first moult at any given time during this period was small.

Second-stage rhabditiform larvae were readily distinguished from those still in the first stage by their larger size, being from 470 to 755$\mu$ long, the greater thickness and obvious striation of the cuticle, the darker intestinal cells, and the conspicuousness of the excretory pore and terminal excretory duct; the latter structures were not seen in first-stage larvae. A slight difference in the structure of the buccal cavity following the first moult was also noted. Comparison of Fig. 4, illustrating the structure of the buccal cavity during the first stage, with Fig. 5, illustrating this structure in an early second-stage larva, shows that in the latter the telostom is definitely present and the prorhabdions and metarhabdions are slightly separated.

Although no free-living nematodes were found in the cultures from which *Nippostrongylus* larvae were obtained, additional cultures were made, as a precautionary measure, from faeces removed from the rectum of infected rats directly to culture dishes. About ten rhabditiform larvae, which had partially extricated themselves from the loosened first cuticle, were transferred individually to separate small glass dishes containing a few drops of water. In this medium the development of the larvae to the third stage readily occurred. This eliminated any possibility that the larvae observed undergoing the first moult might have been those of a nematode other than *N. muris*.

The essential features of the larval development following the first moult are substantially as described by Yokogawa in his discussion of the "first moult". In the light of the writer's findings, Yokogawa's "first moult" is actually the second moult, as suggested by Schwartz & Alicata (1935) on the basis of the morphology and behaviour of the infective larva. According to Yokogawa, changes in the structure of the oesophagus precede modification of

the structure of the posterior extremity of the larva during this ecdysis. The writer's observations of *N. muris* larvae in the second ecdysis indicate, and this appears to be a general rule in the development of strongyle larvae, that transition from the long tail of early second-stage larvae to the short tail characteristic of infective larvae is well advanced or completed before changes in the rhabditiform structure of the oesophagus are initiated.

Although Yokogawa's description of the "first larval stage" includes morphological data and size relationships pertaining to preinfective larvae of both preparasitic stages, publication of detailed descriptions of larvae of the first and second stages is not deemed essential because the structure of preinfective strongyle larvae is rarely, if ever, of value for purposes of specific identification. The morphological features already mentioned are adequate for the differentiation of first-stage and second-stage larvae of *N. muris* as usually encountered in cultures.

The infective larva of *N. muris*, which was formerly considered, from the standpoint of development, a second-stage larva is a true third-stage larva. Although Yokogawa has described the infective larva in considerable detail, certain features of the finer structure of the anterior and posterior extremities are included in this paper because of the possible importance which the structure of third-stage larvae may ultimately assume in the classification of nematodes.

The elliptical mouth opening of the third-stage larva is surrounded by four prominent cephalic papillae, and is followed by a very narrow, short, cuticularised canal leading to a vestibulum, the walls of which appear in dorsal or ventral view as two short parallel cuticular rods to which are attached fine muscle fibres extending from the termination of the oesophagus; posteriorly the vestibulum connects with the lumen of the oesophagus. Viewed dorsally or ventrally, the amphids appear as two small lateral pores or pouches located slightly posterior to the lips; from these pores fine ducts pass internally and posteriorly as shown in Fig. 6. In lateral view, the amphidial canal resembles a question mark in shape. Typically the caudal extremity of the larva terminates in three conical processes, a large dorsal one and two smaller subventral ones. Phasmids are located slightly anterior to the caudal tip.

## COMPARATIVE STUDY OF THE CUTICLE OF ADULTS OF *NIPPOSTRONGYLUS MURIS* AND OTHER TRICHOSTRONGYLES

Although the writer's findings on the preparasitic development of *N. muris* have cleared up the problem of the number of preparasitic moults, Yokogawa's view that an incomplete moult occurs, following what would ordinarily be regarded as the final moult, requires consideration. According to Yokogawa, *Nippostrongylus* has an outer, thick, transversely striated cuticle with longitudinal ridges and an inner, new, thin cuticle with transverse striae but without longitudinal markings. Yokogawa states that after the completion of the

second parasitic moult, the cuticle becomes looser and looser and a new cuticle similar to that produced in moulting develops under the old one, the latter finally separating over most of the body from the new underlying cuticle. This produces a relatively wide space between the two cuticles. These observations are regarded by Yokogawa as indicating the occurrence of an incomplete post-adult moult.

While a confirmation or rejection of Yokogawa's observations might be based entirely upon a careful re-examination of the cuticular structure of *N. muris*, it appeared to the writer that an interpretation of the peculiarities of the cuticle of this species might logically be based upon a comparison of the cuticle of *N. muris* with that of other trichostrongyles. Sections of preserved adults of *Haemonchus contortus*, *Oswaldocruzia* sp., *Longistriata musculi*, *L. noviberiae* and *Nippostrongylus muris* were stained with Mallory's triple stain (haematoxylin, orange G and anilin blue). While the colour of homologous tissues in the species under consideration differed somewhat in various preparations, the latter were satisfactory for study.

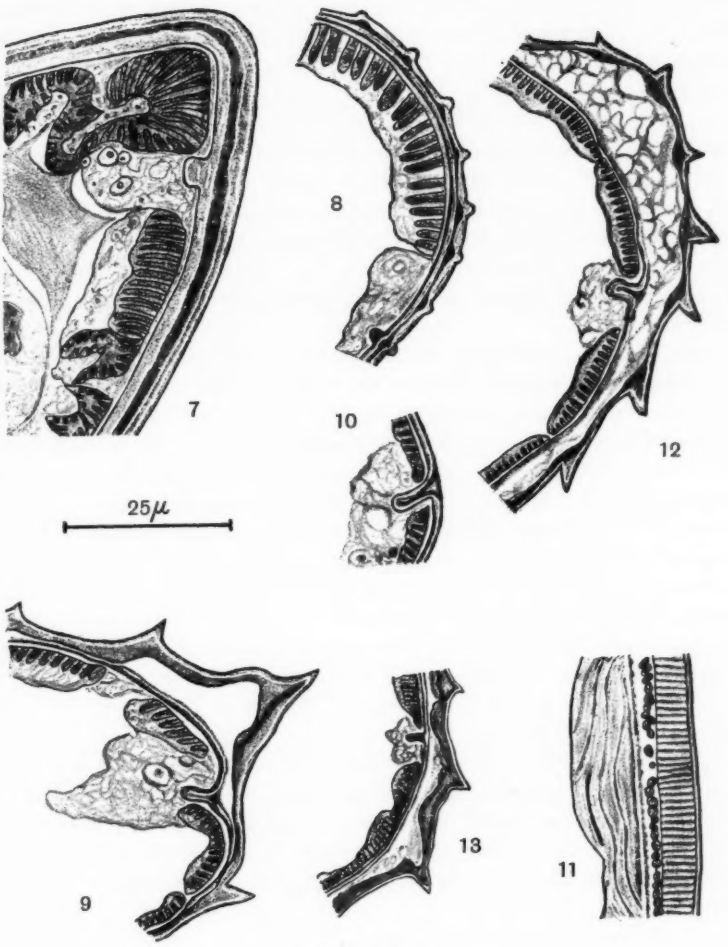
### The cuticle of *Haemonchus contortus*

This species was selected for study because the work of Veglia (1916) has shown definitely that four moults occur during the development of *Haemonchus*.

The cuticle of *H. contortus* forms a number of external longitudinal ridges, as is evident either from examination of the surface of the cuticle of preserved specimens or from a study of cross sections of the worm. Cross sections showed the structure of the cuticle to be as follows: Externally the cuticle was bounded by the darkly stained tunica externalis; internal to this membrane there were present the following layers, not sharply separated from one another, but showing differential staining characteristics: (1) a thin, almost unstained, superficial layer; (2) a thin, light blue layer; (3) a thicker, dark blue layer; (4) a thick, internal layer stained light blue, often with a greenish tinge, which showed more or less evidence of separation into two layers in the region of the lateral chords. This last layer, bounded internally by the tunica internalis, appeared to extend without differentiation in the form of a fold or internal ridge ("chitinous rods") into the region of each lateral chord. The hypodermis appeared as a thin faintly staining layer between the tunica internalis and the external edges of the muscle cells (Fig. 7).

### The cuticle of *Oswaldocruzia* species

The cuticle of this worm is much thinner than that of *Haemonchus contortus*, and it is impossible to state definitely the precise number of layers of which it is composed. The cuticle forms a large number of external longitudinal ridges, apparently between fifty and sixty. Between the tunica externalis and the tunica internalis at least three cuticular layers, showing differential staining properties, occur (Fig. 8). Internal lateral ridges extending into the lateral




Figs. 7–13.

Fig. 7. *Haemonchus contortus.* Cross-section in region of lateral chord, showing cuticular layers.
Fig. 8. *Oswaldocruzia* species. Cross-section in region of lateral chord, showing cuticular layers.
Fig. 9. *Nippostrongylus muris.* Slightly oblique cross-section in region of lateral chord, showing cuticle, hypodermis and musculature.
Fig. 10. *N. muris.* Oblique cross-section in region of lateral chord (the cuticular inflation is intentionally omitted) showing internal ridge formed by invagination of the tunica internalis and two internal cuticular layers.
Fig. 11. *N. muris.* Longitudinal section through cuticle, hypodermis and musculature.
Fig. 12. *Longistriata musculi.* Slightly oblique cross-section in region of lateral chord, showing cuticle, hypodermis and musculature.
Fig. 13. *L. noviberiae.* Cross-section in region of lateral chord, showing cuticle, hypodermis and musculature.

chords are present in *Oswaldocruzia*. These ridges appeared to be formed by folds of the tunica internalis and the basal cuticular layer, as is the case in *Haemonchus*.

### The cuticle of *Nippostrongylus muris*

It is obvious, even at low magnifications, that cuticular inflation occurs in *N. muris*, not only in the cephalic region, but also over the remainder of the body anterior to the vulva in the female and anterior to the bursa in the male. The cephalic inflation is without longitudinal markings or definite transverse striae. The striated post-cervical inflation bears, as is shown in cross sections, fourteen or fifteen longitudinal ridges, rather than ten as has been previously reported.

Cross sections in the region of the post-cervical inflation showed the structure of the cuticle to be as follows: externally the cuticle was bounded by the darkly stained tunica externalis, internal to which the following layers appeared: (1) a very thin, faintly stained, superficial layer; (2) a much thicker greyish or greyish-blue layer; (3) a thin, intensely stained, dark blue layer, differentiated from the tunica internalis with difficulty except in the region of the lateral chords, and separated from the first two cuticular layers by a wide orange-staining area or a space containing orange-staining globules; (4) a thin, less intensely stained, basal layer, distinguishable from the third layer only in the region of the lateral chords (Fig. 9). The internal lateral ridges of the cuticle ("chitinous rods") occurring in the lateral chordal regions were sharply set off internally from the hypodermis and appeared to be formed by folds or invaginations of the tunica internalis, the thin basal layer, and the darkly stained third layer (Fig. 10).

The double layering of the cuticle comprising the post-cervical inflation was confirmed by examination of longitudinal sections. The striation of the second layer was extremely marked; the presence of the thin, homogeneous first layer was indicated by the fact that the striae did not quite extend externally to the tunica externalis (Fig. 11). Layer 3, internal to the orange-staining area, showed no evidence of transverse striation. Some evidence of the presence of layer 4 was seen in longitudinal sections.

Living female specimens, immobilized in egg albumen, were examined in optical section. The post-cervical inflation was seen to consist of two cuticular layers and the cephalic inflation to consist of the superficial layer only, the striated, second cuticular layer terminating anteriorly at the place where the cephalic inflation commenced. The post-cervical inflation is separated from the internal cuticular layers by a space containing a reddish fluid. In the region of the cephalic and post-cervical inflations, and posterior to the vulva, at least two internal cuticular layers occur. The character of the third cuticular layer did not differ in any of these regions and there was no evidence that this layer is transversely striated, as stated by Yokogawa. By cutting off the posterior end of female specimens a short distance anterior to the vulva and pushing the

inflated portion of the cuticle back over the tail, it was possible to examine the surface of the third cuticular layer on these small fragments of the worm without the optical interference which is caused by the presence of the inflation when entire mounts of the living worms are examined. The examination revealed no evidence of transverse striation, but the surface of this layer was marked with a large number of very fine longitudinal rows of inverse punctations.

### THE CUTICLE OF *LONGISTRIATA MUSCULI* AND *L. NOVIBERIAE*

As Dikmans (1935) has noted in his description of these two species, the cuticle is inflated not only in the cephalic region but, as in *Nippostrongylus muris*, is inflated also over the body and here is marked by longitudinal ridges. The structure of the cuticle in these species is essentially the same as in *N. muris*. Cross sections showed that the post-cervical inflation is composed of two cuticular layers. In *Longistriata musculi* (Fig. 12) the cuticle of the inflation is thinner than in *L. noviberiae* (Fig. 13), and the two layers are identified principally by differential staining properties. Internal lateral cuticular ridges occur in the region of the chords in both species. These ridges are apparently produced by folds of the tunica internalis and the basal cuticular layer. In *L. musculi* evidence was seen of the occurrence of two cuticular layers internal to the space beneath the post-cervical inflation. The most internal of these two layers was the one most involved in the formation of the internal lateral ridges ("chitinous rods").

### DISCUSSION ON THE STRUCTURE OF THE CUTICLE IN TRICHOSTRONGYLES

Study of the cuticle of *Haemonchus contortus* indicates that the cuticle of this species is composed of at least four distinct layers and that the longitudinal internal ridges ("chitinous rods") occurring in the regions of the lateral chords are formed by folds of the tunica internalis and the basal cuticular layer. So far as could be determined, the cuticle of *Nippostrongylus muris* in particular, but also of the other trichostrongyles studied, is homologous with that of *Haemonchus contortus*. No evidence was discovered that the cuticular layer internal to the post-cervical inflation in *Nippostrongylus muris* is striated or has any of the characteristics of a newly formed cuticula. The writer's observations, interpreted in the light of the findings in *Haemonchus contortus*, indicated, on the contrary, that this layer, in both *Nippostrongylus* and *Longistriata*, is homologous with the third cuticular layer of *Haemonchus*.

In contrast to these findings, Veglia (1916) considers the cuticle of *Haemonchus contortus* as composed of a single layer, although certain of his figures clearly show two cuticular layers external to the "subcuticle" or hypodermis. According to Veglia, "chitinous rods" appear at the base of the lateral bands or chords, that is, between the cuticle and hypodermis. Veglia considers these

structures as distinct from both the cuticula and the hypodermis. Looss (1905) states that the cuticle of *Ancylostoma duodenale* is composed of two layers, not always divided, however, by a sharp line. Looss, also, seems to consider that the "chitinous rods" are to be differentiated from the cuticle, and states that they are not firmly fused with it. According to him, when there is a tendency for the layers to separate, the "chitinous rods" usually adhere to the hypodermis rather than to the cuticle, but he could find no membrane separating the "chitinous rods" from the basal cuticular layer.

Even if the cuticular nature of the "chitinous rods", which are considered by the writer to be folds of the basal cuticular layer in the species studied, is regarded as a subject requiring further investigation, these structures are at least intimately connected with the basal cuticular layer in strongyles, and in cross sections afford a clue to the identity of the proximal cuticular layer.

It is concluded that in *Nippostrongylus muris* the post-cervical inflation of the cuticle is produced by the separation of the second and third layers of a unit four-layered cuticle such as is found in *Haemonchus contortus*, there being no evidence of the formation of a new underlying cuticula or of the occurrence of an incomplete post-adult moult.

In the light of this work, the development of *Nippostrongylus muris* involves four complete moults and does not differ in any essential respect from the development reported for other strongyloid nematodes.

## Summary

Rhabditiform first-stage larvae of *Nippostrongylus muris* are about 270–550$\mu$ long. The development of these larvae culminates in a moult, which occurred 36–48 hours after cultures were prepared. The first moult is preceded by an unusually early separation of the first cuticle, which is fine and unstriated, from the body tissue in the tail region and, later, by the formation of an underlying new cuticle, which is thicker and prominently striated.

Early second-stage rhabditiform larvae differ in certain morphological respects from first-stage larvae. Second-stage larvae are about 470–750$\mu$ long.

A second preparasitic ecdysis occurs and the infective larva is a true third-stage larva. This moult was the only one previously known to occur during the preparasitic development of *N. muris*.

The post-cervical cuticular inflation in *N. muris* is regarded, on the basis of a comparative study of the cuticle of this species and of *Haemonchus contortus* and other trichostrongyles, as resulting from the separation of the second and third layers of the cuticle. Yokogawa's view that *Nippostrongylus muris* has two cuticulae is rejected. *N. muris* has a single cuticula, apparently made up of four layers.

*N. muris* undergoes four complete moults and the development of this species does not differ in any essential respect from that reported for other strongyles.

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# CRITERIA FOR THE ACCURATE CLASSIFICATION OF THE RICKETTSIAL DISEASES (RICKETTSIOSES) AND OF THEIR ETIOLOGICAL AGENTS

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(With Plates IV and V, containing Figs. 1–10)

## Introduction

Certain arthropod-borne diseases of man and lower animals are rather arbitrarily but usefully classed as "Rickettsial diseases". The term "Rickettsiosis" has recently been introduced into the literature, as a synonym for "Rickettsial disease". These diseases are caused by minute but non-filterable bacterium-like micro-organisms, characteristically intracellular in habitat, which multiply in their arthropod vectors as well as in their mammalian hosts. A large number of morphologically similar but non-pathogenic intracellular micro-organisms have been described in arthropods, and it has been suggested that the etiological agents of the rickettsial diseases may be members of this group, and that their pathogenicity for mammals may be an accidental phenomenon.

Excluding trench fever and dengue, which have not been transmitted to laboratory animals and in which the etiological agents have not been established with certainty, this group includes typhus, spotted fever, tsutsugamuchi disease, and heartwater. The latter is a disease of cattle and sheep and has not been reported in man.

In recent years, acute febrile diseases of man in nearly all parts of the world have been recognised as belonging to the rickettsial group. These diseases show considerable variation in clinical picture, both in man and in experimental animals. The occurrence of mild and atypical forms appears to be an outstanding characteristic of this group of diseases, and a characteristic which makes their differential diagnosis a difficult matter.

Attempts to classify these diseases on the basis of clinical pictures in man and laboratory animals, with or without casual morphological studies, have led to a number of unsatisfactory and unjustifiably complex classifications which are quite inconsistent with one another. In many instances, unidentified diseases suspected of belonging to this group have been transmitted to the guinea-pig, and the causative organism has been found in smears of scrotal sac exudate, but more complete morphological studies have not been made

The tendency to assign a local or personal name to organisms seen in such incompletely studied strains is unfortunate, since it has often led to distinctions without differences and to the accumulation of invalid terms in the literature.

Accurate classification of these clinically vagarious diseases can, we believe, be based only upon careful morphological, histopathological, and immunological studies. Methods for studies of this type have been described in detail by Wolbach (1919) and Wolbach, Todd & Palfrey (1922).

The accurate classification of the bacterial diseases has usually awaited the completion of cultural studies of the organisms involved and histopathological studies of the lesions produced by them. By these methods, widely varying clinical pictures have frequently been unified. The organisms which cause the rickettsial diseases have not been cultivated outside of cells, but their study in tissue culture (Pinkerton & Hass, 1931, 1932 *a*, *b*, *c*) is an almost equally valuable morphological method, since one may easily obtain large numbers of heavily infected cells in which the organisms are as well stained as ordinary bacteria. This method has also been found valuable for determining the intranuclear or intracytoplasmic location of the organisms, a point of primary importance in the differential diagnosis between typhus and spotted fever. Intranuclear localisation of the spotted fever organism was shown by Wolbach (1919) to occur under natural conditions in the tick, but has been found in mammalian tissues only after *in vitro* cultivation at 32° C. for at least 8 days.

Previous to 1927, the problem of differentiation between the rickettsial diseases was not recognised, since the various members of this group appeared to be easily distinguished from one another on the basis of geographical distribution, host specificity, and clinical pictures. Recent work has largely destroyed geographical barriers and has shown that typhus has a rodent reservoir, and that the rat flea as well as the louse may be its vector to man. When typhus and spotted fever occur in the same communities, practically identical clinical pictures may be produced, both in man and in experimental animals. Focal brain lesions, previously believed to be pathognomonic of typhus, have been found to occur in mild strains of spotted fever. These facts have greatly complicated the situation, and made it necessary to study each unknown strain completely by all available methods before attempting to classify it.

In our experience, when such complete morphological, histopathological and immunological studies have been made, difficulties in classification have invariably disappeared, and each of the seven diseases which we have studied in this laboratory has been identified in a clean-cut manner as typhus or spotted fever. There is considerable reason for believing that the mite-borne diseases (of which tsutsugamuchi disease is the prototype) form a third group, but definite proof of this has not yet been presented.

The objects of this paper are to define and tabulate the criteria on which it is believed that an accurate classification may be based, to summarise

classificatory data obtained by their use in this laboratory, and to classify all reported rickettsial diseases and their etiological agents on this basis, in so far as available data will permit. The absence of important data makes this classification purely tentative in many instances, but the attempt seems worth while, if only to show where further data are needed.

The breaking down of clinical and geographical barriers between typhus and spotted fever has led several authors to suggest that these two diseases may have a common parentage, and that intermediate types may occur. Our laboratory studies have entirely failed to support this assumption but have, on the contrary, emphasised the fundamental differences between the two diseases, as well as the essential identity of the various strains of each disease.

Before going further, we will describe and tabulate the criteria which we consider important in the differential diagnosis and classification of these diseases.

### Criteria for classification

(1) *Cross-immunity tests.* For practical purposes this is perhaps the most important single criterion and, correctly carried out and interpreted, such tests will often serve to identify an unknown strain beyond a reasonable doubt. The requisites are known virulent strains of typhus and spotted fever. Having established the unknown disease in guinea-pigs, recovered animals from the two known strains are inoculated along with control animals. Animals which have reacted definitely to the unknown disease are also inoculated with the known strains of typhus and spotted fever. It is desirable to wait a month after complete defervescence before reinoculating to test for immunity, and of course always to reinoculate with an adequate amount of virulent material. A rare source of error is the injection of the material directly into a distended intestine, in which case infection frequently fails.

When dealing with a strain of feeble virulence (and it is in feebly virulent strains that problems of differentiation usually arise) it may be necessary to employ a large number of animals in order to get a satisfactory result. In a strain of mild spotted fever from Minnesota which we recently studied (Pinkerton & Hass, 1932 *c*), less than 80 per cent. of normal guinea-pigs reacted definitely. Of fourteen animals which did react definitely, only five were completely immune to Rocky Mountain spotted fever. Eleven animals, recovered from the latter disease, however, showed no reaction to the disease under investigation. Only twelve out of twenty-three typhus-immune guinea-pigs reacted definitely to the disease under investigation. Eight animals, recovered from the disease under investigation, all reacted to endemic typhus, but in six instances with a delayed incubation period and mild fever.

We regarded these results as strongly suggestive of the identity of our unknown strain with spotted fever, but they were inconclusive. The final identification of this strain was based on the morphological study of its causative organism in smears, sections of guinea-pig testicle and scrotum, and in tissue cultures (see below).

In cross-immunity tests between two strains of a disease which are essentially identical, but which vary markedly in virulence, we may expect the more virulent one always to protect completely against the less virulent one, but are not surprised if the protection afforded by the less virulent one against the more virulent one is incomplete.

Very little importance can be attached to the production of "partial" immunity, since we have found that marked lengthening of the incubation period, reduction in the height of fever, and at times apparently complete failure to react to endemic typhus, may be brought about by the intraperitoneal injection of large doses of non-pathogenic air contaminants which grew on blood agar plates. Even the injection of large amounts of normal brain tissue was found to have a definite effect, lasting for several weeks[1]. This source of error can be partly, but not entirely, overcome by inoculating with overwhelming doses of virus, but, when dealing with feebly virulent strains, results must be interpreted with caution.

Incompletely reciprocal cross-immunity is, we believe, entirely consistent with the identity of two strains which vary greatly in virulence. In our cross-immunity tests we have gone on the assumption that a fully virulent typhus strain should protect completely against all strains of typhus, and that a fully virulent strain of spotted fever should protect completely against all strains of spotted fever. We have never found a strain which will produce any evidence of infection in both typhus-immune and spotted fever-immune guinea-pigs, when the immunity has developed as the result of a typical reaction to fully virulent strains. A strain producing typical infection in both typhus-immune and spotted fever-immune guinea-pigs would necessarily be regarded as belonging to neither group. With the possible exception of tsutsugamuchi disease, the immunological aspects of which will be discussed later, no evidence has been found that such a strain exists.

(2) *Study of smear preparations from the scrotal sac exudate.* The severity of the acute inflammatory reaction occurring in the scrotal sac varies greatly with different strains of rickettsial disease. It is mildest in European (epidemic) typhus and in Rocky Mountain spotted fever, but occasional animals reacting to both of these viruses may show it in a severe form. The number of infected cells varies with the severity of the reaction and with the stage at which the examination is made. The pattern of cell infection, the types of cell invaded, and the morphology of the organisms, however, we have found to be independent of these factors and of differential diagnostic value.

In typhus exudates we have invariably found the majority of infected cells packed with organisms so that they may be readily identified under the low-power lens (Fig. 1). This has been true of all strains of typhus which we have studied.

[1] It is of interest to note that Nicolle (1935) recently found a considerable number of guinea-pigs refractory to typhus following vaccination against *B. paratyphosus* B and the bacillus of pseudo-tuberculosis.

The staining method described by Castaneda (1930) is superior to the Giemsa method for identifying these heavily infected cells under the low power, since, in a well-stained preparation, such cells stand out as rounded bright blue structures against the red background formed by non-infected and lightly infected cells. This method is more difficult, however, and less reliable than the Giemsa method, so that it is advisable to use both methods.

In the case of those strains which we have placed in the spotted fever group, organisms are diffusely scattered in the cell cytoplasm, the majority of cells containing less than fifteen organisms and the number of organisms per cell rarely exceeding twenty-five. Examination with high-power lenses is necessary in order to locate these cells (Fig. 2).

We have shown (Pinkerton, 1931 *a*) that in typhus exudates only the serosal cells become infected with organisms. In the two strains of spotted fever which we have employed for this purpose, we found that macrophages (identified by ingestion of graphite ink particles) also harbour organisms.

The morphology of the organisms in the two diseases is also distinctive, we believe. Comparisons are best made in Giemsa-stained smears, selecting lightly infected cells in the case of typhus, in order to make conditions comparable, since the size of the organisms appears to depend definitely on the number present in a cell. Under these conditions, spotted fever organisms appear larger, more bluish in staining reaction, much more often lanceolate or diplococcoid in shape, and surrounded by a more definite halo (Fig. 2).

We believe that the differential diagnosis can always be made from smear preparations on the basis of these criteria, although individual cells from the two diseases may at times be indistinguishable.

(3) *Histopathology.* Human autopsy material is difficult to obtain in mild atypical rickettsial diseases, but is in many ways inferior to guinea-pig tissues for diagnostic purposes.

As we have already noted, focal brain lesions can no longer be regarded as having much differential value. In guinea-pigs we have been unable to distinguish the small focal lesions of vascular origin which occur in Eastern spotted fever from those of typhus. In a human case of probable Eastern spotted fever, however (erroneously reported by Pinkerton & Maxcy (1931) as endemic typhus because spotted fever was believed to be ruled out on geographical grounds), acute fibrin thrombi with "numerous polymorphonuclears" were found in the vessels, and it was noted that "this feature was more marked than in the usual case of European typhus". Circumscribed areas of "hyaline degeneration of the white matter without cellular reaction" were also noted, as well as perivascular zones of vacuolisation which were regarded as infarcts. These features were later noted and fully described by Lillie (1931) in known human cases of Eastern spotted fever, and undoubtedly are of diagnostic importance, when present.

The most important organs for study in the guinea-pig are the scrotum, scrotal sac, epididymis, and testis. The characteristic lesion of spotted fever

is the formation of acute fibrin thrombi in the arterioles and venules of these tissues. These thrombi contain polymorphonuclears and pyknotic nuclear fragments and may wholly or partially occlude the lumen (Fig. 9). Polymorphonuclears are also frequently present in the walls of the affected arterioles and venules, so that the reaction might be considered as a destructive panangeitis, in contrast to the purely proliferative endangeitis of typhus. In mild strains, such lesions may not be numerous, but we have found them without great difficulty in all strains which we have classed as spotted fever, and never in any strain of typhus, in spite of prolonged search.

Endothelial proliferation of capillaries, arterioles and venules is common to both diseases and without differential value.

Compact perivascular collections of lymphocytes and macrophages (Fraenkel's nodules) are more pronounced in typhus than in spotted fever. (See Fig. 10 and accompanying description.)

It should be noted that the line of demarcation between the proliferative endangeitis of typhus and the thrombonecrosis of spotted fever is less sharp in human tissues (especially in sections of human skin) than it is in guinea-pig tissues. In sections of skin excised from living patients, it may be impossible to differentiate between spotted fever and typhus on this basis alone, but the location and morphology of the causative organism (see below) is of crucial value.

Histopathological studies on a number of guinea-pigs reacting to an unknown disease may then be expected to yield evidence of importance in differential diagnosis, thrombonecrosis in arterioles and venules being characteristic of spotted fever and never found in typhus.

(4) *Location and morphology of organisms in sections.* Either human or guinea-pig tissues may be used for these studies, but the latter are more readily available, and equally satisfactory. Fixation in Regaud's fluid and staining by the Giemsa method give the best results. Sections from the scrotum, testicle, epididymis, and cremasteric muscle should be prepared from several guinea-pigs.

We have recently found that a constantly maintained *p*H of 7·2 is optimum for the distilled water before adding the stock solution of Giemsa stain, since this greatly facilitates differentiation. Phenol red is added to the distilled water and the reaction is adjusted to the above *p*H. The stain is then added, the slides immersed in an overflowing staining jar which is then immediately sealed with a paraffined rubber stopper to prevent absorption of $CO_2$ from the air.

In typhus one finds mesothelial cells lining the tunica vaginalis which are packed with enormous numbers of organisms (Fig. 5). These cells are found readily except in cases where the scrotal sac exudation is inconspicuous. In the vascular endothelium of the above tissues organisms may rarely be found after prolonged search, but smooth muscle cells in the vessel walls are never invaded.

In spotted fever, the mesothelial cells and macrophages in the scrotal sac

exudate show the same sparse infection pattern which has been described in smear preparations. The distinctive and diagnostic lesion of spotted fever is the presence of numerous organisms in the smooth muscle cells, as well as in the endothelial cells of the arterioles and venules in the above tissues (Fig. 6). This picture was first described by Wolbach (1919) in Rocky Mountain spotted fever and was recognised by him as an important diagnostic criterion. It has been readily found in all strains of rickettsial disease which we have diagnosed as spotted fever and is usually found with very little difficulty. Organisms are most numerous in vessels which show the thrombonecrotic lesions described above.

(5) *Study of the organism in tissue culture.* The technique for studies of this type has been previously described (Pinkerton & Hass, 1931, 1932 *a*, *b*, *c*). It is relatively simple, and the great majority of explants show infection of practically every cell present at the end of two weeks.

In the absence of scrotal sac exudate, spleen may be used as explant material. We have used this successfully in Rocky Mountain spotted fever, but our attempts in European typhus were not successful (possibly because of the inability of the typhus organism to grow within phagocytic cells). In the great majority of rickettsial diseases, scrotal sac exudate is formed and is the material of choice. Even when no external evidence of a scrotal reaction is present, exudate will often be found if the guinea-pig is killed on the first day of fever.

In tissue cultures, the contrast between the typhus and the spotted fever cell infection patterns is a striking one (Figs. 7 and 8). In typhus, the cytoplasm of the cells is packed and often greatly distended with organisms. Nuclei are never invaded, and occasionally are seen to be uniformly contracted and pyknotic as a result of the cytoplasmic distention. In spotted fever, the cytoplasm is relatively sparsely infected, while the nuclei contain spherical masses of organisms, of variable size, but often entirely filling and greatly distending the nucleus. These intranuclear forms are often coccoid, and so minute as to be barely within the range of visibility. Occasionally the intranuclear clusters appear homogeneous.

The organisms themselves also show well-marked morphological differences. In addition to the differences described in smear preparations, one finds, in typhus, long twisted threads (up to 60 $\mu$ in diameter) composed of diplobacilli joined end to end. This picture, which is characteristic of freshly infected cells, where conditions for multiplication are ideal, has not been found in spotted fever. In dying cells, typhus organisms appear as minute eosinophilic coccoid bodies, while spotted fever organisms appear as dark blue staining bacilliform or lanceolate bodies, of relatively large size. (In spotted fever cultures, we have occasionally found deeply stained organisms which approach the size of ordinary bacteria. We believe that this appearance is the result of overstaining, since these organisms have in several instances resumed normal size after further decolorisation.)

On the basis of the above criteria, typhus may be accurately and definitely separated from spotted fever by the tissue culture method. Among several hundred well-infected cultures, representing three strains of each disease, intermediate or ambiguous pictures have not been found. We regard the tissue culture method as of equal value with the sectioning of infected arthropods in establishing a definite diagnosis.

(6) *Study of organism in arthropod vector.* In mild epidemics, in which the vector is unknown, but in which the disease has been established in guinea-pigs, tissue culture studies should be made, and we may be guided by the results obtained in our search for the arthropod vector. Strains of lice, fleas, ticks, or mites, known to be free from virus and, if possible, free from micro-organisms of any sort, should be obtained. Ticks are readily infected with spotted fever by feeding on infected guinea-pigs, by the technique described by Wolbach (1919). Rat-fleas and rat-lice may be infected by feeding on infected rats (Mooser & Castaneda, 1932). Human lice may be fed upon infected monkeys, or infected intrarectally by injecting blood from typhus patients (Weigl, 1924) or scrotal sac exudate (Mooser & Dummer, 1930). Infected mites occur in nature (Hayashi *et al.*, 1933), and presumably these arthropods could be experimentally infected, but we have been unable to find any publications presenting the cell infection pattern in the mite. Sections of infected mites seem at present to be the most important material for study of these diseases.

Table I. *Differential diagnosis between typhus and spotted fever in the arthropod vector*

| | Typhus | Spotted fever |
|---|---|---|
| Arthropod vectors | Insecta:<br>Man to man by human body-louse (*Pediculus humanus*)<br>Rat to man by rat-flea (*Xenopsylla cheopsis*)<br>Rat to rat by rat-louse (*Polyplax spinulosus*) and rat-flea | Arachnoidea:<br>Mammal to mammal by several species of tick, notably *Dermacentor andersoni, D. variabilis* and *Amblyomma cajennense* |
| Reaction of arthropod vector to infection | Lice die in a few weeks; rat-fleas recover | Ticks survive and infection is hereditary |
| Location of organism in arthropod vector | Intracytoplasmic—confined to cells lining the alimentary tract | Intracytoplasmic and intranuclear. Invades all tissues |
| Morphology of organism in arthropod vector | Identical with that seen in tissue cultures (see text) | Identical with that seen in tissue cultures (see text) |

The principal differences between typhus and spotted fever in arthropod vectors are shown in Table I. When ticks are dissected (Wolbach) and stained by the Regaud-Giemsa method, the intranuclear organisms are brought out with striking clearness. The hereditary nature of the infection in the tick, and the invasion of all organs of the tick by the organisms, are also phenomena distinctive of spotted fever.

The contrast between the cell infection pattern in the typhus louse (and typhus flea) and that in the spotted fever tick is shown in Figs. 3 and 4.

(7) *Weil-Felix reaction.* We have had no experience with this test in experimental animals, but it is included in our criteria for the sake of completeness and because there seems reason to believe that it will prove of value in differential diagnosis.

Felix (1933) has made elaborate studies by this method, and believes that viruses of the typhus group show "main" agglutination (*i.e.* agglutination in high titre) for the O × 19 strain of *B. proteus*, and that the mite-borne viruses show "main" agglutination for the O × K strain, while viruses which we class in the spotted fever group show only "group" (low titre) agglutination for both strains.

The results of these studies, with one exception, are quite consistent with and confirmatory to our own studies. The exception is the case of São Paulo "typhus", which, on the basis of morphological and immunological studies by Toledo Piza and others (1932), belongs definitely to the spotted fever group, but which showed "main" agglutination for O × 19, differing from the typhus group only in the presence of "group" agglutinations for O × K.

The possibility of double infection of certain arthropod vectors should be borne in mind and further studied in the laboratory, but evidence at present indicates complete specificity of each virus for its arthropod vectors. Zinsser & Castaneda (1931) found that the typhus virus could remain alive in ticks for 14 days, but obtained no evidence that it was transmissible by the bite of the tick.

Felix (1933) points out that serological studies are necessary for the accurate classification of this complex group of diseases. We agree with this, but would be inclined to ascribe major importance to morphological and histological methods.

It should be noted that Felix (in company with many European investigators) refers to all rickettsial diseases as "typhus", a point of view which, in view of our sharp separation of true typhus from the diseases which we class as spotted fever, we feel should be abandoned. Without insisting on the name "spotted fever" we feel that some distinguishing name is necessary for this well-demarcated group of diseases.

(8) *Clinical picture in the guinea-pig.* Considerable overlapping has been found in clinical features previously believed to be valuable in the differential diagnosis. Identical pictures of injection and oedema of the scrotum, accompanied by exudation into the scrotal sac, have been found in several mild strains of both spotted fever and typhus, but this reaction, in typhus, occurs only after intraperitoneal inoculation. Virulent strains of spotted fever produce necrosis, wrinkling and dry gangrene of the scrotum (a phenomenon never seen in typhus) and, in typical Rocky Mountain spotted fever at least, this reaction occurs not only after intraperitoneal inoculation but also after subcutaneous inoculation into the skin of the back. Animals inoculated by this route with various strains of typhus never show any gross involvement of the scrotum, tunica vaginalis or testes. Many strains of spotted fever have a low or high mortality in the guinea-pig, whereas no strain of typhus has been

shown to have any mortality (secondary infection must of course be ruled out to make this observation of value). Thus the clinical picture in the guinea-pig may establish the diagnosis in virulent strains of spotted fever, but is of no value in distinguishing mild strains of spotted fever from typhus.

Table II. *Differential diagnosis between typhus and spotted fever in the guinea-pig*

| | | Typhus | Spotted fever |
|---|---|---|---|
| Clinical picture | Mortality | 0 | 0 to 100 % |
| | Loss of weight | 0 to + | 0 to ++++ |
| | Scrotal redness and swelling | 0 to ++++ | 0 to ++++ |
| | Scrotal necrosis and gangrene | 0 | (+) to ++++ |
| Gross pathology | Spleen | + | ++ |
| | Scrotal sac exudation | 0 to ++++ | 0 to ++++ |
| Histology of vascular lesions in scrotum, testis, epididymis, and cremasteric muscle | | Endothelial proliferation in arterioles, capillaries, and venules. Thrombosis of capillaries by proliferated endothelial cells | Same as typhus but more marked, and with the addition of thrombonecrosis in arterioles and venules—a distinctive spotted fever lesion |
| Focal brain lesions | | + to ++++ (polymorphonuclears rare) | 0 to ++ (polymorphonuclears common) |
| Morphology of organisms | In Giemsa-stained smears of scrotal sac exudate | Diplobacilli, rarely in short chains, packing cells, purple staining, relatively small, found only in serosal cells | Diplobacilli and lanceolate diplobacilli, scattered sparsely in cytoplasm and surrounded by a clear zone, bluish staining, relatively large, found in serosal cells and macrophages |
| | In sections Regaud fixation, Giemsa stain | Closely packed in serosal cells of tunica vaginalis. Very rarely seen in small numbers in vascular endothelium in scrotum and in brain lesions | Found sparsely in serosal cells and macrophages. Easily found in moderate numbers in endothelial and *smooth muscle cells* of vessels in scrotum, testis and brain |
| | In tissue cultures (at 32° C.) of scrotal sac exudate (Regaud-Giemsa) | Cytoplasm becomes packed, *nuclei never invaded.* A diplobacillus, occurring in chains up to 30 $\mu$ in length. Minute eosinophilic globular forms in dying cells | Cytoplasm relatively sparsely infected. *Nuclei packed and distended.* Diplobacilli and lanceolate diplococcoid forms. Blue staining lanceolate form (relatively large) in dying cells |
| Cross-immunity | With known typhus strains | Complete | Negative except for delay of incubation (non-specific) and decreased mortality |
| | With known spotted fever strains | Negative | Complete with strains of high virulence. Incompletely reciprocal (see text) with strains of low virulence |

(9) *Clinical picture in man.* We include this criterion also for the sake of completeness. The epidemiological and clinical features of diagnostic importance were well described by Badger, Dyer & Rumreich (1931) in connection with the simultaneous occurrence of the two diseases in the south Atlantic states. There is, however, ample evidence that clinical features alone are of little value. São Paulo "typhus" for example has been definitely established as spotted fever, and evidence is accumulating that *fièvre boutonneuse*, with its "tache noir" is also a variety of spotted fever, although it was regarded as typhus on clinical grounds.

## Discussion

The criteria described above will, it is believed, serve for the identification of any unknown rickettsial disease (if transmissible to laboratory animals) as belonging to the typhus group, to the spotted fever group, or to a third group, the existence of which has not been conclusively demonstrated. The criteria are tabulated in Tables I and II.

The seven strains which we have studied by these methods in this laboratory have been grouped as shown in Table III. The Minnesota strain could not be classified on the basis of the clinical picture in man or in the guinea-pig, but was definitely identified as spotted fever on the basis of the above criteria. In spite of its mild and atypical nature, it did not seem expedient to regard this as a new disease, or its etiological agent as a new variety of organism even though, in the guinea-pig, it produced a clinical picture definitely different from that of Rocky Mountain spotted fever and that of Eastern spotted fever.

Table III. *Classification of rickettsial diseases studied in this laboratory*

| | |
|---|---|
| Typhus | European (epidemic) strain (Wolbach) |
| | European (epidemic) strain (Breinl) |
| | Mexican (endemic) strain (Mooser) |
| | North Carolina (endemic) strain (Maxcy) |
| Spotted fever | Rocky Mountain strain (Parker) |
| | Eastern strain (Badger, Dyer & Rumreich) |
| | Minnesota strain (Reimann) |

Morphological studies in this laboratory on one strain of *fièvre boutonneuse* (Mediterranean fever) are as yet incomplete, but, with other available evidence, justify placing this disease tentatively in the spotted fever group. The recent histological studies of Gomes (Toledo Piza, Meyer & Salles Gomes, 1932), together with the immunological studies of Dyer (1933) and of Parker & Davis (1933), place São Paulo "typhus" very definitely in the spotted fever group.

The studies of Nakajima (1932) seem to indicate that tsutsugamuchi is immunologically distinct from typhus and spotted fever, but are inconclusive because of the relatively low susceptibility of the rabbit to these diseases. Monkeys could probably be used for more accurate cross-immunity experiments between these three diseases, since definite infection in this animal is regularly produced with all three viruses. The histological studies of Ogata, Unno & Nakajima (1930) indicate that the histiocytes of the testis of the rabbit become infected after intratesticular inoculation of the virus, but leave unanswered the question of infection of smooth muscle cells in arteriolar walls. Infection of histiocytes is suggestive of the spotted fever group of organisms, as is the fact that the mite belongs to the same class (Arachnoidea) as the tick, a different class from the louse and the flea (Insecta).

Table IV represents an attempt to classify all reported rickettsial diseases, on the basis of the above criteria. In the interest of conciseness, only those

Table IV. *Classification of all reported rickettsial diseases*

Cross immunity

Organism in

Organism in

Arthropod

Organism in

Weil-Felix main

Diagnosis and

**Table IV.** *Classification of all reported rickettsial diseases*

| Name | Cross immunity: With typhus | Cross immunity: With spotted fever | Histopathology | Organism in mammalian tissues | Organism in tissue cultures | Arthropod vectors | Organism in arthropod vectors | Weil-Felix main agglutinins | Diagnosis and remarks |
|---|---|---|---|---|---|---|---|---|---|
| *European (human) (classical) (epidemic) typhus | Complete | Slight non-specific (?) | Proliferative endangeitis | In endothelium and mesothelium | Intracytoplasmic; distends cytoplasm; never in nuclei | *Pediculus humanus* | Distends cells lining gut. Never invades nuclei or viscera. Not hereditary | O × 19 | Typhus |
| *Tabardillo Mexican (endemic?) typhus | Do. | Do. | Do. | Do. | Do. | *Xenopsylla chaeopis*; *Polyplax spinulosus*; (*Pediculus humanus*)? | Do. | O × 19 | Typhus |
| *Endemic typhus in south-eastern U.S.A. | Do. | Do. | Do. | Do. | Do. | *Xenopsylla chaeopis*; *Polyplax spinulosus* | Do. | O × 19 | Typhus |
| Manchurian typhus | Do. | ?? | ?? | ?? | ?? | Do. | Do. | ?? | Typhus |
| Endemic typhus (Australia) | ? | ?? | ?? | ?? | ?? | ?? | ?? | O × 19 | Typhus? |
| Ship fever (Toulon) | ?? | ?? | ?? | ?? | ?? | *Xenopsylla chaeopis*? | ?? | O × 19 | Typhus? |
| Tropical typhus. Type W. "Shop" typhus | Complete | ?? | ?? | ?? | ?? | ?? | ?? | O × 19 | Typhus? |
| Brill's disease: Usually regarded as identical with Tabardillo and endemic typhus in south-eastern U.S.A. Believed by Zinsser, on statistical and experimental evidence, to represent recrudescence of childhood infections with European virus | | | | | | | | ?? | Typhus |
| *Rocky Mountain spotted fever | Slight, non-specific? | Complete | Thrombonecrosis in arterioles and venules | In endothelium and smooth muscle cells of vessel walls. Rarely in mesothelium | Intranuclear; distends nuclei; sparse infection of cytoplasm | *Dermacentor andersoni*. Other species of ticks? | Intranuclear and intracytoplasmic. Invades all tissues. Hereditary | Neither O × 19 nor O × K | Spotted fever |
| *Eastern spotted fever | Do. | Do. | Do. | Do. | Do. | *Dermacentor variabilis* Other ticks? | ?? | ?? | Spotted fever |
| *Minnesota strain (Reimann). Clinically doubtful | Do. | Incompletely reciprocal | Do. | Do. | Do. | ?? | ?? | ?? | Spotted fever |
| São Paulo "typhus" | Do. | Complete | Do. | Do. | ?? | *Amblyomma cajennense*. Other ticks? | Invades all tissues | O × 19 | Spotted fever (Weil-Felix reaction ambiguous) |
| **Fièvre boutonneuse* | Do.? | Complete (?) | Thrombonecrosis (?) | Do.? | Intranuclear. Distend nuclei. Sparse infection of cytoplasm? | *Rhipicephalus sanguineus*. Other ticks? | ?? | Neither O × 19 nor O × K | Spotted fever? Morphological studies in this laboratory incomplete |
| Tick-bite fever (South Africa) | ? | ? | ?? | ?? | ?? | Ticks | ?? | Neither O × 19 nor O × K | Spotted fever? |
| Tsutsugamuchi disease | Negative? | Negative? | ? | Infects histiocytes of rabbit testis | ? | *Trombidium akamushi*. Other arachnids? | ?? | O × K | More like spotted fever than typhus. A third disease? |
| Tropical typhus. Type K. "Scrub" typhus | ?? | ?? | ?? | ?? | ?? | Mites | ?? | O × K | Identical with tsutsugamuchi? |

?? means that no report on this observation can be found. ? means that observations are ambiguous or inconclusive.

* means that this disease has been studied in this laboratory.

criteria of outstanding importance are included in this table, which is intended only as a temporarily useful guide to the understanding of the problems involved in clarifying the present chaotic situation.

In many instances, a large number of different names have been applied to entirely similar diseases in different parts of the world. It seems probable that clinical differences observed in the same disease in different regions depend upon two factors.

(1) Variation in virulence alone which, in the case of the spotted fever group especially, may be so great as to cause marked clinical differences. Since all degrees of virulence are found in various local epidemics, it is obvious that separation into varieties cannot be made upon this basis alone.

(2) Variations in arthropod vectors and in intermediate hosts. Endemic or murine typhus seems to be identical with epidemic or human typhus (Pinkerton, 1931) except for slight changes in biological properties of the virus, presumably the results of residence in the rat and in the ectoparasites of that animal. The author found that the Wolbach strain of European typhus, after passage through rats, acquired the ability of producing, temporarily, the severe scrotal reaction characteristic of the endemic or murine strain. Mooser (1932) later obtained the same result by passing the European virus through rats and fleas, as did Varela & Gay (1934) still more recently by rat passage alone.

In a group of diseases with such complex host relationships as those under investigation, it seems probable that a large number of strains might arise which are biologically somewhat modified. It is doubtful, however, whether these strains should be considered as new varieties unless these biological modifications are definitely and constantly demonstrable in the laboratory. It is probable that the spotted fever group of diseases will be found to have one or more strains which are at least as sharply differentiated from its prototypal form (Rocky Mountain spotted fever) as is murine from human typhus.

One must guard against indiscriminate "lumping" because of the fact that important differences may be overlooked, comparable to those which exist between *M. tuberculosis humanus* and *M. tuberculosis bovis*. It is interesting, in connection with the question of differences between human and murine typhus, to note that several workers have suggested the possibility that bovine tubercle bacilli may be transformed into the human type by prolonged residence in a human being. In so chronic a disease, the chances for variations as a result of adaptation would be excellent. It must be remembered, however, that the separation of typhus organisms into human and murine types rests on a much less secure foundation than the separation of tubercle bacilli into human and bovine types.

In considering the problem presented by the classification of these diseases and of their etiological agents, it is realised that nomenclature is built up by usage, and that unnecessary changes from established custom are undesirable.

It is obvious, however, that the present system of nomenclature contains many terms which are invalid.

Any classification proposed at present must be purely tentative because of the incompleteness of available data. On morphological grounds, it seems necessary at present to consider the causative organisms of this group of diseases as closely allied to bacteria and widely separated from Protozoa. Their exact relation to bacteria must await further study, but we may perhaps regard them tentatively as forming a bacterial family.

Reasons for the sharp separation of these diseases into two main groups (typhus and spotted fever) have been given above. The well-established name for the etiological agent of typhus fever is *Rickettsia* (genus) *prowazeki* (species). Such names as *Rickettsia manchuriae* (n.sp.), *Rickettsia mooseri*, etc., must be discarded, since they have been applied to organisms which definitely do not belong to different species but which are at most varieties, and in many cases are even identical with one another.

*Rickettsia prowazeki* was the name given by da Rocha-Lima to the etiological agent of classical, louse-borne (European, epidemic) typhus. Only one variant of this organism has thus far been described. The etiological agent of rat-flea-borne (murine) typhus was first seen in enormous numbers by Mooser in Mexican typhus (tabardillo) in the scrotal sac of guinea-pigs, and has been shown to differ from the organism causing classical typhus in several minor but definite biological features[1]. If a name is required to distinguish it from the latter organism, a logical one would be *Rickettsia prowazeki mooseri*, since it is at most a variety. The etiological agents of Manchurian (endemic, rat-flea-borne) typhus, of the entirely similar disease which occurs along the Atlantic seaboard in the United States, and of entirely similar diseases in practically all other parts of the world, should obviously be given the same name.

The complete absence of overlapping between the distinguishing morphological and histopathological characteristics of the etiological agents of typhus and spotted fever forces one to place them in different species and justifies one in placing them in different genera.

The name given by Wolbach to the etiological agent of spotted fever, following his complete and accurate morphological studies, was *Dermacentroxenus rickettsi*. The validity of the genus *Dermacentroxenus* was questioned by Brumpt (1927) who preferred to place the spotted fever organism and *R. prowazeki* in a single genus. (The name proposed by Brumpt was *Rickettsia rickettsi*.) This author did not mention the intranuclear localisation of the spotted fever organism nor the important morphological differences between it and *Rickettsia*

[1] It produces a febrile disease in the rat, while classical *Rickettsia prowazeki* produces only unapparent infection. Injected per anum (in controlled doses?) it kills *Pediculus humanus* in a few days, the classical variety requiring about three weeks. It produces scrotal sac exudation more constantly than the classical strain. Minor immunological differences between the two organisms have been noted by Zinsser.

*prowazeki*, and regarded the etiological relationship of the organism to spotted fever as not definitely established, in spite of the very conclusive evidence presented by Wolbach.

It is therefore doubtful if Brumpt realised the important morphological and physiological differences between the two organisms. Recent studies in tissue culture have emphasized and extended these differences. For these reasons it is suggested that the original name *Dermacentroxenus* (genus) *rickettsi* (species) be retained for the organism and that it, together with *R. prowazeki*, be considered tentatively as belonging to one family (Rickettsiaceae). If further study should show that sufficient strain differences exist to justify the addition of variety names, the organism of São Paulo "typhus" would become *Dermacentroxenus* (genus) *rickettsi* (species) *braziliensis* (variety) etc.

The diseases themselves will undoubtedly retain their local or "place" names to a large extent, but little confusion need arise from this, as long as scientific workers have a clear conception of their identity with the typhus group or with the spotted fever group, and of the precision with which these two groups may be separated from one another.

Our suggested classification of the etiological agents of the rickettsial diseases is presented in Table V.

Table V. *Classification of the etiological agents of the rickettsial diseases*

| Family | Genus | Species | Variety | Habitat and remarks |
|---|---|---|---|---|
| RICKETTSIACEAE | *Rickettsia* | *prowazeki* (Rocha Lima) | *prowazeki* (nom.nov.) | Cause of epidemic (classical) typhus in man. Occurs in *Pediculus humanus corporis* and in *P. humanus capiti* |
| | | Do. | *mooseri* (nom.nov.) | Cause of endemic (murine) typhus in man. Occurs in wild rats, rat-fleas, and rat-lice. Separated from *R. prowazeki* on basis of secondary biological modifications |
| | | *tsutsugamuchi* (orientalis) | — | Cause of tsutsugamuchi disease, probably present in rats and in harvest mite (*Trombidium akamushi*). Separation from *R. prowazeki* and *D. rickettsi* not yet on an entirely firm basis |
| | | *ruminantium* (Cowdry) | — | Cause of "heartwater" in cattle and sheep. Found in ticks |
| | | *pediculi* (*wolhynica*, *quintana*) | — | Occurs extracellularly in gut of louse. Hypothetical cause of trench fever |
| | | Unnamed | — | Occurs in *Aedes aegypti*. Hypothetical cause of dengue |
| | *Dermacentroxenus* | *rickettsi* (Wolbach) | — | Cause of a number of diseases in man, all belonging to S.F. group. It occurs in several species of ticks, in which it is transmitted hereditarily. A large variety of mammals probably act as intermediate hosts. Disease produced in man variable in severity and in clinical picture, but specific varieties not yet recognised. Diseases include Rocky Mountain spotted fever, Eastern spotted fever, a clinically undiagnosed disease in Minnesota (referred to in text), São Paulo typhus, and probably also *fièvre boutonneuse* and tick-bite fever of South Africa |

## SUMMARY AND CONCLUSIONS

Morphological, histopathological, and immunological studies of seven strains of rickettsial disease have placed them in two widely separated groups, for which typhus and spotted fever seem the most logical names.

Diseases of the spotted fever group are carried by ticks (Arachnoidea) and characterised by distinctive histopathological lesions—thrombonecrosis of arterioles and venules, with micro-organisms of definite and characteristic morphology, invading smooth muscle cells, as well as endothelium, mesothelium, and macrophages. Tissue culture studies show massive invasion and distention of cell nuclei, while cytoplasmic invasion of host cells is relatively sparse.

Diseases of the typhus group are carried by lice and fleas (Insecta) and characterised by the facts that the intracellular parasite which causes them invades only endothelium and mesothelium, distends the cytoplasm of its host cells without ever invading nuclei, and in guinea pigs causes only proliferative endangeitis without thrombonecrosis.

In the spotted fever tick, organisms are intranuclear as well as intracytoplasmic, invade nearly all types of tissue, and are transmitted hereditarily. In typhus-infected lice and fleas, organisms are intracytoplasmic only, infect only the lining cells of the gut, and are not transmitted hereditarily.

Neither focal brain lesions nor scrotal sac exudation is of importance in the differential diagnosis, since both occur in certain strains of spotted fever, as well as in typhus. Definite necrosis of the scrotum is of importance, since it is dependent upon the above-described arteriolar thrombonecrosis and is peculiar to spotted fever.

A review of the literature shows that rickettsial diseases and their etiological agents are being classified in an inaccurate and unjustifiably complex manner, specific names often being given to organisms without the presentation of evidence sufficient to establish them as new varieties.

A plea is made for the study of unclassified or unsatisfactorily classified diseases in all parts of the world by the methods described, and for their classification on the basis of morphologic and histopathologic observations on insect and mammalian hosts and of immunological studies on the latter, rather than on variations in clinical pictures in man and in experimental animals.

The criteria for the differential diagnosis between typhus and spotted fever are presented in tabular form and illustrated wherever possible. All reported rickettsial diseases as well as their etiological agents are classed on the basis of the criteria presented, and, with the exception of the incompletely studied mite-borne group, the available evidence strongly suggests that all are varieties of typhus and spotted fever.

The morphological, histopathological, and immunological differences between typhus and spotted fever are so great that these two diseases, as well

as their causative organisms, should be widely separated from one another, and given definitely distinguishing names.

It is suggested that the well-established name, *Rickettsia prowazeki*, be applied to intracytoplasmic micro-organisms in Insecta causing all diseases of the typhus group, and that *Dermacentroxenus rickettsi* (the name originally given by Wolbach) be applied to hereditarily transmitted micro-organisms invading nuclei in Arachnoidea and in tissue cultures, causing all diseases of the spotted fever group. Specific varieties, when established by the demonstration of definite and constant biological differences, should be indicated by variety names, as *Rickettsia prowazeki mooseri* (for endemic flea-borne typhus in all parts of the world).

ACKNOWLEDGMENT

We wish gratefully to acknowledge the co-operation of the originators of the various strains of rickettsial diseases (Wolbach, Breinl, Mooser, Maxcy, Parker, Badger, Dyer, Rumreich, and Reimann), without which these studies could not have been made.

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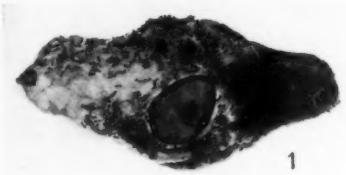

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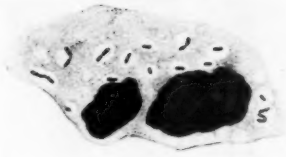

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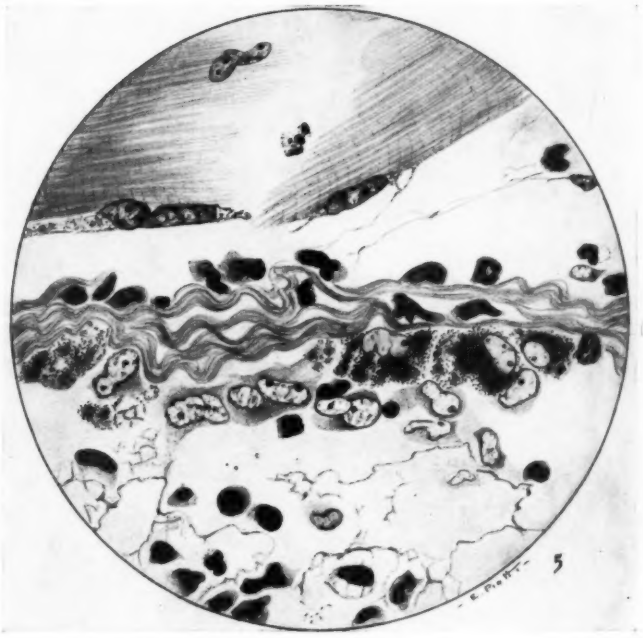

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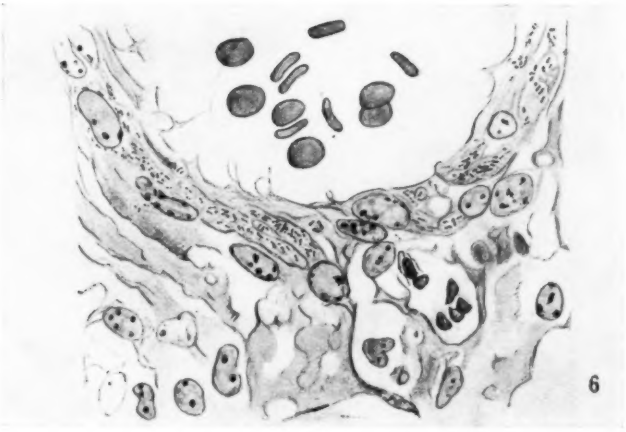

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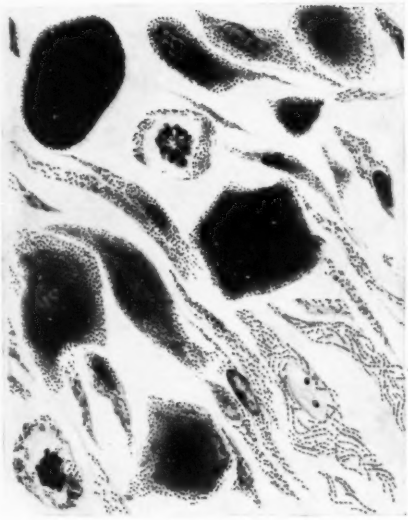

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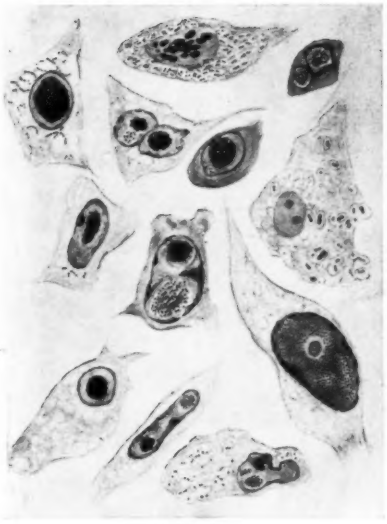

Fig. 8.

Fig. 9.

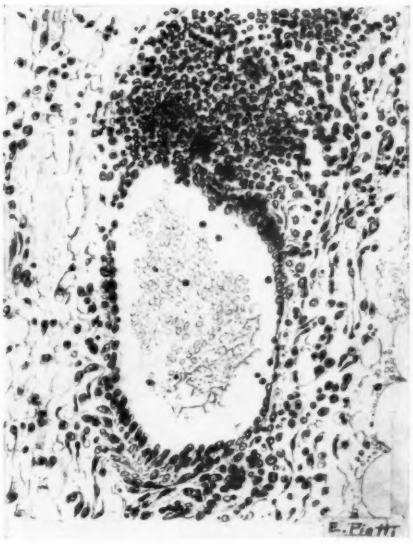

Fig. 10.

## EXPLANATION OF PLATES

### PLATE IV

Fig. 1. Typhus. Giemsa-stained smear of scrotal sac exudate from guinea-pig reacting to an endemic (Maxcy) strain. Mesothelial cell typically infected with *Rickettsia prowazeki*, showing characteristic morphology and cell infection pattern. In more heavily infected cells (which are frequently encountered) individual organisms cannot be resolved, and the cytoplasm is uniformly dark in appearance. (Camera lucida drawing.)

Fig. 2. Spotted fever. Giemsa-stained smear of scrotal sac exudate from guinea-pig reacting to the "Eastern" strain. Mesothelial cell typically infected with *Dermacentroxenus rickettsi*, showing characteristic morphology and cell infection pattern. To be compared with Fig. 1.

Fig. 3. Typhus (European). Infected louse (*Pediculus humanus*). Paraffin section, Zenker-fixed, Giemsa-stained. Three mesothelial cells lining the anterior diverticulum are included, the central cell having its cytoplasm greatly distended with *R. prowazeki*.

Fig. 4. Spotted fever (Rocky Mountain). Infected tick (*Dermacentor andersoni*). Paraffin section. Regaud-fixed, Giemsa-stained. Three mesothelial cells lining the Malpighian tube are shown. The central cell shows intranuclear massing of *D. rickettsi* and consequent swelling and degenerative changes in the nuclear membrane. The two adjacent cells show normal nuclei. A few scattered organisms are present in the cytoplasm. This picture of nuclear distension was seen and described by Wolbach in 1919. It is never seen in infected arthropod vectors of diseases of the typhus group. (Camera lucida drawing.)

Fig. 5. Typhus. Regaud-fixed, Giemsa-stained paraffin section through the scrotal sac of a guinea-pig reacting to an endemic (Maxcy) strain. *Rickettsia prowazeki* in cytoplasm of mesothelial cells. Striated muscle of scrotum above. Exudate in lower part of field. This is the characteristic localisation and cell infection pattern for all strains of typhus.

Fig. 6. Spotted fever (Rocky Mountain). Regaud-fixed Giemsa-stained-paraffin section through testis. The smooth muscle cells in the wall of a venule in the polar fat are characteristically infected with *Dermacentroxenus rickettsi*. This picture is characteristic of all strains of spotted fever, and never found in typhus.

### PLATE V

Fig. 7. Typhus (Endemic-Maxcy). Typical appearance of a heavily infected tissue culture, initiated from scrotal sac exudate. Regaud-fixed Giemsa-stained paraffin section from a culture 68 days *in vitro*, transferred five times, but without addition of fresh tissue or virus. Heavily infected cells, packed and distended with diplobacilloid and diplococcoid forms, but no nuclear invasion. Lightly infected cells (lower left) contain threads composed of diplobacilli joined end to end. Note the cell infection pattern in the two cells undergoing mitosis. (Camera lucida drawing. Two microscopic fields have been combined in order to bring out the complete morphological range.)

Fig. 8. Spotted fever (Eastern). Typical appearance of heavily infected tissue culture from scrotal sac exudate, 38 days *in vitro* with four transfers. Technique same as that used for typhus section illustrated in Fig. 7. Shows typical morphology and intranuclear massing of organisms with formation of spherical clusters (rarely almost homogeneous) wrinkling of nuclear membranes, and peripheral condensation of nuclear chromatin. The cytoplasm is sparsely infected. This picture is to be contrasted with that of typhus (Fig. 7). (Camera lucida drawing. Cells have been assembled from three fields to show morphological range.)

Fig. 9. Spotted fever. Section of testis of a guinea-pig reacting to a virulent Rocky Mountain strain. Zenker-fixation and eosin-methylene blue stain. A venule illustrating the thrombo-necrotic type of lesion which is characteristic of spotted fever. Note the fibrin thrombus in the lumen, and the presence of numerous polymorphonuclears in this thrombus and in the vessel wall.

Fig. 10. Typhus. Section of scrotum of guinea-pig reacting to an endemic (Maxcy) strain. Zenker-fixation and eosin-methylene blue stain. A venule showing endothelial swelling and proliferation, and a perivascular accumulation of macrophages and lymphocytes (Fraenkel's node). This lesion is more characteristic of typhus than of spotted fever, but is of slight value in differential diagnosis.

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# ENTRY OF *FILARIA* LARVAE INTO THE BODY CAVITY OF THE MOSQUITO

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(With Plate VI and 3 Text-figures)

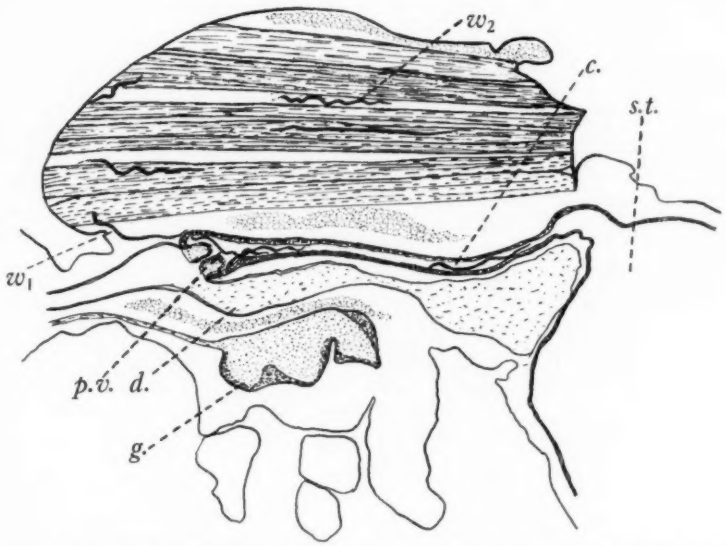
The commonly accepted view regarding the manner of entry of larvae of *Wuchereria bancrofti* (Cobb.) into the body cavity of its mosquito host is that microfilariae from human blood enter the stomach of the mosquito along with the blood meal and after casting their sheath in the stomach, they penetrate through the wall of the stomach, enter the haemocele and then find their way into the thoracic muscles. The time taken for the penetration of the stomach wall according to Looss (1914) is 6–12 hours. According to Bahr (1912) the worms appear in the thoracic muscles in 24 hours, while Lebredo (1905) recorded that in exceptional cases, worms were seen in the thorax 13 hours after feeding, but ordinarily the time interval was longer.

The author working with *Culex fatigans* Wied., experimentally infected with *Wuchereria bancrofti*, and with *Mansonia* (*Mansonioides*) *annulifera* Th. similarly infected with *Filaria malayi* Brug, has observed that in both instances the worms enter the haemocele of the thorax of the mosquito in a remarkably short time, frequently in less than an hour after the infective meal. These studies are based on the examination of serial sections of bred mosquitoes fed on carriers with microfilariae and subsequently fixed at consecutive intervals of 10 min.

The manner in which the worms effect their entry into the haemocele of the mosquito is different from the commonly accepted view on the subject. I find that when a mosquito sucks blood containing microfilariae, the worms that pass up the oesophagus tend to stay in the cardiac portion of the midgut immediately behind the proventriculus (Text-fig. 1). This portion of the mid-gut which is situated within the thorax has a narrow lumen. By forming strong loops against the wall of the gut, the microfilaria stays in the cardiac region against the flow of the blood into the "stomach". It then travels forwards until it reaches the proventriculus. Microfilariae have frequently been observed in this position in sections of mosquitoes fixed within an hour after feeding. It has been further observed that the sheath of a microfilaria remaining in the cardiac mid-gut is softer than normally and shows a tendency to disintegrate. This is probably the effect either of the salivary fluid on the microfilarial sheath or of the secretion of the cells of the proventriculus. It would appear that the microfilaria penetrates through the wall of the gut near the proventriculus, or in the proventriculus itself, and escapes into the perivisceral cavity of the thorax[1].

[1] In this article, the worm in the alimentary canal of the mosquito with the microfilarial sheath intact is called the microfilaria; it is called the worm-larva when it has cast its sheath and entered the haemocele of the mosquito.

While doing so, it leaves behind it in the cardiac mid-gut its partially disintegrated sheath. I have, however, not yet obtained any sections in which the worm is seen partly within the proventriculus and partly outside it in the haemocele. Many specimens have been observed within the proventriculus and immediately outside the proventriculus in the perivisceral cavity of the thorax. This has been observed both in *Culex fatigans* fed on blood with *Wuchereria bancrofti* and in *Mansonia* (*Mansonioides*) *annulifera* fed on blood with *Filaria malayi*. In both instances the worms were seen in the haemocele within a



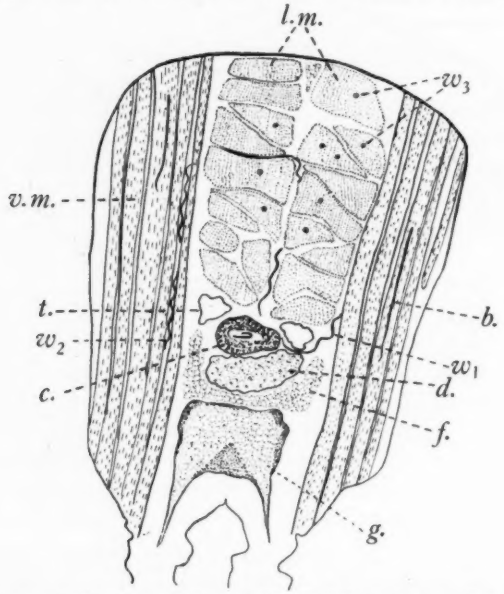

Text-fig. 1. Vertical longitudinal section through *Culex fatigans* fixed 1 hour after feeding on blood with *Wuchereria bancrofti*, showing microfilariae in cardiac mid-gut (*c.*) and proventriculus (*p.v.*), and *Filaria* larvae in the perivisceral cavity of thorax ($w_1$), between the muscle bundles ($w_2$) and inside muscle bundles. (Semi-diagrammatic, reconstructed from serial sections.) *d.* diverticulum; *g.* thoracic ganglia; *s.t.* pyloric mid-gut.

remarkably short time after the meal. No worm-larvae were seen in the haemocele of the abdomen, although they were fairly common in the haemocele of the thorax.

The entry of the worms into the haemocele of the mosquito may occur during or immediately after feeding. In many instances, worms were seen in the perivisceral cavity and in between the muscle bundles within an hour and in a few cases within 20 min. of feeding. It would appear that the penetration of the gut wall is achieved mostly during the first 2 hours after feeding. In observations made with mosquitoes fed on the same carrier and dissected at varying intervals after feeding, it has been observed that the average number of filariae in the thorax increases steadily till 2 hours after feeding, after

which period there does not appear to be any appreciable increase in the number of worms in the haemocele although the stomach contained microfilariae.

These observations show that the region of penetration of the gut wall by the microfilaria is not the "stomach" but the cardiac portion of the mid-gut, especially the proventriculus. In none of the sections of mosquitoes examined in this series were any worm-larvae seen in the haemocele of the abdomen, although a large number were seen in the perivisceral cavity of the thorax. In the narrow lumen of the cardiac mid-gut, the microfilariae have the purchase to penetrate the gut wall by forming strong coils against the wall, while in the



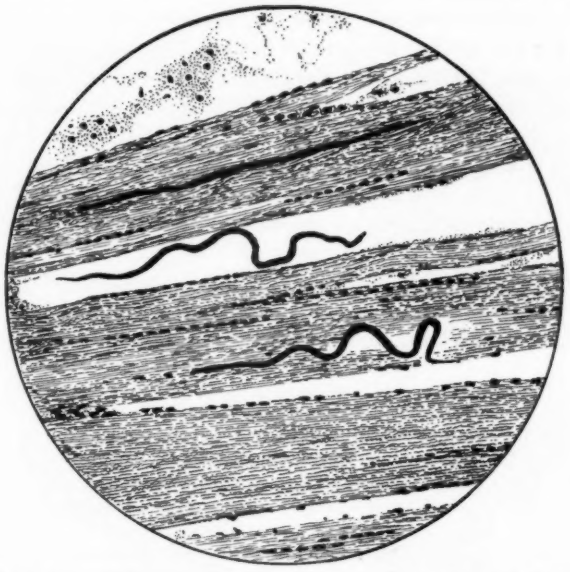

Text-fig. 2. Transverse section through thorax of *Culex fatigans* 1 hour after feeding on blood with *Wuchereria bancrofti*. (Semi-diagrammatic.)

*b.* stretched worm larva in muscle bundle; *c.* cardiac mid-gut with microfilaria; *d.* oesophageal diverticulum; *f.* fat-body; *g.* thoracic ganglia; *l.m.* longitudinal muscles of thorax; *t.* trachea; *v.m.* vertical muscles of thorax; $w_1$, worm larva in perivisceral cavity; $w_2$, worm larva moving between muscle bundles; $w_3$, worm larvae within muscle bundles (in section).

large cavity of the pyloric region such purchase is not available. It seems unlikely therefore that microfilariae could penetrate through the wall of the stomach as has been previously supposed. The short interval between the time of feeding and the occurrence of worm-larvae in the thoracic muscles, the presence of microfilariae on the cardiac portion of the mid-gut and the proventriculus during and soon after feeding, the presence of many worm-larvae in the perivisceral cavity of the thorax and their absence in the haemocele of the abdomen all indicate that the principal point of penetration of the gut by the microfilariae is in the region of the proventriculus and not in the stomach.

Such of the microfilariae that do not stick to the cardiac region of the mid-gut and are driven down into the pyloric region, do not appear to have much chance of entering the haemocele unless they happen to creep up into the cardiac region from the pylorus. Microfilariae, frequently with their sheaths intact, have been observed in the pyloric region for long periods, sometimes as long as 2 days, after a meal, without effecting an entry into the haemocele. This indicates that a microfilaria that comes into the "stomach" does not have much chance of entry into the body cavity of the mosquito.

Fülleborn (1927) mentions that the stomach contents of a mosquito soon after feeding shows a higher concentration of microfilariae than in the peripheral



Text-fig. 3. Section through thoracic muscle bundles showing different stages in the penetration of the muscle bundle by the worm larva 60 min. after feeding. At centre, worm-larva moving in space between muscle bundles; below, a worm-larva penetrating into a muscle bundle; above, a larva that has entered a muscle bundle and has stretched out lengthwise.

blood[1], and he thinks that this is due to a thickening of the blood as a result of the absorption of the blood plasma through the stomach wall of the mosquito, leaving the blood thicker than in the human carrier. Exact information is not available whether the thickening of the blood in the stomach of the mosquito soon after feeding is due to the absorption of the plasma through the stomach wall or to a process of coagulation of the blood caused by the digestive enzymes. Contrary to the views expressed by Fülleborn, however, I find that the concentration of microfilariae in the stomach contents of a mosquito

[1] It is possible that this original observation of Fülleborn was made with the dog filaria *Dirofilaria immitis* and not with *Wuchereria bancrofti*.

which is susceptible to the infection is generally less than in the human carrier. This is due to the fact that all the microfilariae that are sucked up by the mosquito do not come into the stomach, and many of them stay in the cardiac region and enter the haemocele without coming into the stomach. This may not happen in the case of *Dirofilaria immitis*, in which the microfilariae get into the Malpighian tubules after passing through the stomach. The present observations are solely with regard to the two species studied, namely, *Wuchereria bancrofti* and *Filaria malayi*.

The perivisceral cavity of the thorax is surrounded on nearly all sides by the muscle bundles of the thorax, laterally by the two sets of vertical muscles and dorsally by the longitudinal muscle bundles (Text-fig. 2). The worm-larva on escaping into the perivisceral cavity from the proventriculus gets into the muscle bundles, either the vertical ones or the longitudinal ones. It moves about in the spaces between the muscle bundles for some time before finally penetrating them. The different stages in the penetration of the muscle bundle by the worms are shown in Text-fig. 3. Until such time as it has secured a suitable position for resting, the worm-larva shows strong coils or loops. When it penetrates into a muscle bundle, it works its way right into the middle of the bundle, stretches out parallel to the muscle strands and then becomes dormant.

I am indebted to Prof. W. Schüffner and Col. Dr S. L. Brug for the facilities for work which they gave in the Institut voor Tropische Hygiene, Amsterdam. Thanks are also due to Mr C. Johnson for the drawings illustrating this article.

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## EXPLANATION OF PLATE VI

Photo-micrographs of sections of mosquitoes fixed 20–90 min. after feeding.

Figs. 1–4. *Filaria malayi* in *Mansonioides annulifera*.

Fig. 1. Longitudinal section of proventriculus and cardiac midgut showing microfilariae. (× 165.)
Fig. 2. Worm larvae moving in space between muscle bundles of thorax. (× 330.)
Fig. 3. Worm larvae penetrating into the muscle bundles. (× 165.)
Fig. 4. Elongated and resting worm larva inside muscle bundle. (× 165.)

Figs. 5–7. *Wuchereria bancrofti* in *Culex fatigans*.

Fig. 5. Section of proventriculus showing microfilaria in section. (× 330.)
Fig. 6. Microfilaria creeping up cardiac midgut. (× 165.)
Fig. 7. Elongated resting worm in muscle bundle. (× 165.)

*mf.* microfilaria.

(*MS. received for publication* 3. viii. 1935.—Ed.)

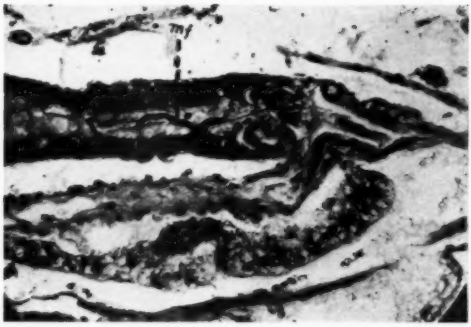


Fig. 1.

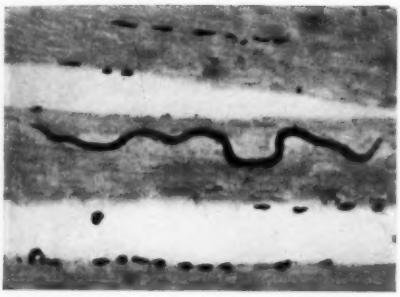

Fig. 2.

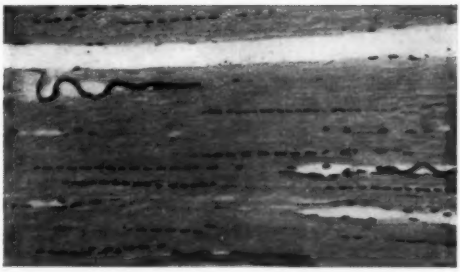

Fig. 3.

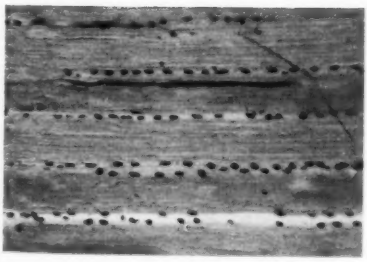

Fig. 4.

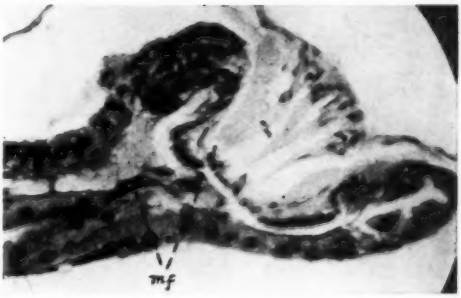


Fig. 5.

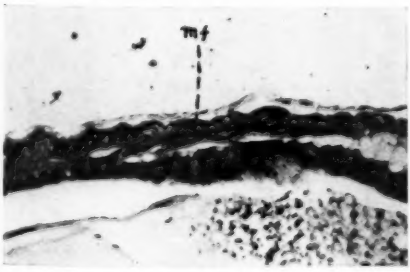


Fig. 6.

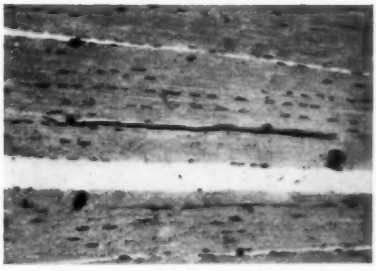

Fig. 7.

# OBSERVATIONS ON THE FLAGELLATES OF THE GENERA *TRICHOMONAS* AND *EUTRICHOMASTIX*

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(With Plate VII, containing Figs. 1–15)

## I. Introduction

The name *Eutrichomastix* was introduced in 1915 by Kofoid & Swezy for the flagellates previously known as *Trichomastix*. The reason for this change was that Vollenhoeven (1878) had already bestowed the generic name *Trichomastix* on a hymenopteron. The flagellates of the genus *Eutrichomastix* differ from those of *Trichomonas* in that they have no undulating membrane, and the number of their flagella is constant, *i.e.* always 4, in all species.

Some observers regard *Eutrichomastix* as a phase in the life history of *Trichomonas* and not a distinct genus. Thus Doflein (1916) believed that the two forms belonged to the same genus. Chatton (1920) found that in cultures the *Trichomonas* of the guinea-pig might assume either a *Trichomonas* or a *Eutrichomastix* form; but that when the latter were injected into the peritoneal cavity of a guinea-pig, *Trichomonas* forms reappeared within a few hours. Reichenow (1918, 1920) noted that occasionally in lizards (*Lacerta muralis* and *L. viridis*) the blood stream was invaded by *Eutrichomastix* from the intestine. In one case in which a lizard had died of such an infection, at the time of death the only forms present in the blood were of the *Eutrichomastix* type. On the next day, however, in addition to these there were other flagellates of the *Trichomonas* type present. Reichenow considers it possible that the latter had been derived from the former, and that the two types may be stages of one organism.

Duboscq & Grassé (1923, 1924) state that there occur *Trimitus*, *Eutrichomastix* and *Trichomonas* stages in the life cycle of *Janickiella grassii*. On the other hand, Dobell (1907) found only *Eutrichomastix serpentis* in the intestinal contents of *Boa constrictor*. He regards *Eutrichomastix* as a separate genus, although he noticed that degenerating *Eutrichomastix* had a tendency to assume a *Trichomonas*-like form owing to the posterior flagellum adhering to the body. Wenyon (1926) has cultivated *Trichomonas* from the tortoise (*Testudo radiata*), the python (*Python molurus*), and the frog, and in none of these did the flagellate have a tendency to assume the *Eutrichomastix* form. He therefore believes that these flagellates should be regarded as belonging to two distinct genera. Bishop (1931) maintained several strains of *Tricho-*

*monas batrachorum* over a period of months, and during this time the flagellate did not undergo any morphological variation. There are also many other workers who prefer to regard *Eutrichomastix* as a separate genus, but conclusive evidence in support of this view is still lacking.

In view of this discrepancy an attempt has been made to ascertain the true position. Reptilian and amphibian hosts have been selected for this work because most species of *Eutrichomastix* hitherto described occur in cold-blooded animals (see Table I, p. 197). Furthermore, it is easier, in most cases, to maintain cultures of flagellates from these sources. The caecal contents of a number of lizards, salamanders, alligators, snakes, etc., were examined. This work was begun in America in winter when most of these animals hibernate and the intestines are almost empty. Out of many such animals examined only one snake gave a good culture of *Trichomonas*. A brief account of this *Trichomonas* has been published elsewhere (Das Gupta, 1935).

## II. Material and methods

The animals used for the present work consist of a krait (*Bungarus candidus*), a chamaeleon (*Chamaeleon quilensis*), 9 English grass-snakes (*Natrix natrix*), and a *Boa constrictor*.

The krait and the chamaeleon were from India and the Cameroons respectively, and died at the London Zoo. The grass-snakes were purchased in London. The culture of the intestinal contents of the *Boa* was received through the courtesy of Prof. E. Reichenow of Hamburg. The intestinal contents of the grass-snakes and the chamaeleon were cultivated on Row's haemoglobin saline medium. This is preferable to Dobell's classical (HSre + S) medium for growing trichomonad flagellates, as it affords a much cleaner background in the stained specimens, although the latter medium yields more copious growth. In the case of *Trichomonas* optimum growth is obtained on the 3rd or 4th day after inoculation, but *Eutrichomastix* grows much more slowly—requiring about a week for a maximum growth. Rice-starch is a very useful adjunct, but it was omitted because the *Trichomonas* ingests the starch granules with such voracity as to make the recognition of their internal structures extremely difficult. *Eutrichomastix* does not seem to ingest unaltered rice-starch. Both these flagellates grow well at 23° C., though they can survive at much higher and lower temperatures. Thin smears of cultures of different stages were stained by alkaline Giemsa's stain following fixation whilst still wet over osmic acid vapour. Some preparations were fixed in sublimate alcohol and stained by Heidenhain's method. Cultures were also examined in the fresh state by dark-ground illumination, which revealed the structure of the flagellates even better than the stained specimens.

In the case of the krait only stained preparations of the intestinal contents were available.

Of the animals examined in the present study *Bungarus candidus* and

Table I

| Species of *Eutrichomastix* | Name of worker and date of publication |
|---|---|
| Reptiles: | |
| *E. lacertae* from species of *Lacerta* | Bütschli (1884). Redescribed by Prowazek (1904) |
| *E. lacertae* from *Lacerta ocellata* | Franchini (1921) |
| *E. viperae* in *Vipera aspis* | Léger (1904) |
| *E. serpentis* from *Boa constrictor* | Dobell (1907) |
| *E. serpentis* from *Pituophis catenifer* (Blainville), *Eutaenia sirtalis* (Linn.) and *Crotalus oregonus* (Holbrook) | Kofoid & Swezy (1915) |
| *E. mabuiae* in the Ceylon lizard, *Mabuia carinata* | Dobell (1910) |
| *Eutrichomastix* sp. in *Hemidactylus leschenaultii* | |
| *Eutrichomastix* sp. in *Dryophis mycterizans* | |
| *E. saurii* in a Brazilian lizard, *Amphisboena* sp. | Fonseca (1917) |
| Amphibians: | |
| *E. batrachorum* in frogs and probably other Amphibia | Dobell (1909, 1910) |
| Fishes: | |
| *E. motellae* in *Motella tricirrata*<br>*E. salpae* from *Box salpa* | Alexeieff (1910) |
| Invertebrates: | |
| *E. trichopterae* from trichopteran larvae | Mackinnon (1910) |
| *Eutrichomastix* from tipulid larvae | Mackinnon (1912) |
| *E. termitis* from termites | Bernstein (1928) |
| *E. rhinocrici* from a myriapod, *Rhinocricus* sp. | Fonseca (1928) |
| Cattle: | |
| *E. ruminantium* | Braune (1913) |
| Guinea-pigs: | |
| *E. caviae* | Grassi (1881) |
| *E. caviae* var. *rossica* | Yakimoff, Wassilewsky, Korniloff & Zwietkoff (1921) |
| *E. caviae* from the wild *Cavia aperea* and the agouti (*Dasyprocta agouti*) of Brazil | Fonseca (1916) |
| Birds: | |
| *E. gallinarum* from fowls | Martin & Robertson (1911) |
| *E. gallinarum* from ducks | Kotlán (1923) |
| Man: | |
| *Trichomastix hominis* | Chatterjee (1917). Probably confused with a small *Chilomastix mesnili* |
| Flagellate of the *Eutrichomastix* type | Haughwout & Horilleno (1920). ? an altered *Trichomonas* |

*Chamaeleon quilensis* were infected with *Eutrichomastix*; *Boa constrictor* was infected with both *Eutrichomastix* and *Trichomonas*; and of 9 specimens of *Natrix natrix* 4 were negative, 3 were infected with *Trichomonas* alone, and 2 with both *Trichomonas* and *Eutrichomastix*.

Using Hegner's technique (described in a previous publication, Das Gupta, 1935) I isolated pure-line strains of *Trichomonas* from a grass-snake and from the *Boa*, and one pure-line strain of *Eutrichomastix* from another grass-snake. In addition to these, stained specimens of the caecal contents of the krait and a culture of *Eutrichomastix* of the chamaeleon were carefully studied.

## III. Description of the flagellates

### (*a*) Trichomonas *from the grass-snake*

The shape of the body is piriform or (under some conditions) rounded. The size varies from $11–17{\cdot}5 \times 4–12\mu$, the average length being $14{\cdot}2\mu$. There are 3 anterior flagella of unequal length. The nucleus lies in the anterior third

of the body and is approximately oval. The axostyle is fairly thick and projects but little beyond the posterior end of the body. The undulating membrane is prominent with 3 or 4 deep waves. The posterior flagellum where it borders the undulating membrane consists of two distinct filaments (Figs. 1–3). This characteristic is constant and typical. The cytoplasm is usually loaded with food inclusions such as bacteria, yeasts, etc. This flagellate readily ingests starch granules introduced into the culture medium.

A strain was studied for a period of 10 weeks, and it showed no tendency to assume a *Eutrichomastix* form.

### (*b*) Trichomonas *from the boa*

Prof. Reichenow has been maintaining a strain of *Trichomonas* mixed with a few *Eutrichomastix* for several years. A pure culture of *Trichomonas* was obtained from his mixed strain and studied for 3 weeks. During this period an occasional individual in the stained preparations simulated a *Eutrichomastix*, owing to separation of the posterior flagellum bordering the undulating membrane; but fresh preparations of the same cultures, examined by dark-ground illumination, never showed *Eutrichomastix* forms. It is therefore evident that this detachment of the posterior flagellum from the body is purely accidental and artificial.

The size varies from $8–15{\cdot}8 \times 4–7{\cdot}6\mu$. The 3 anterior flagella are of unequal length. The axostyle is slender and protrudes beyond the body for a distance of about $4–6\mu$ or even more (Figs. 4–6). It is of uniform thickness. The nucleus is slightly oval and measures $3 \times 2{\cdot}5\mu$. The undulating membrane has one or two shallow waves and is liable to become detached (Fig. 6) in spreading the film. During the course of this study it was impossible to detect any sign of a basal fibre, and it seems probable that the structure is lacking. The cytoplasm shows a large number of food vacuoles with bacterial inclusions.

### (*c*) Eutrichomastix *from the krait*

About 400 individuals were examined in half-a-dozen slides. All the forms encountered were typical *Eutrichomastix*.

The shape varies from long slender forms to short stout ones. The size ranges from $9–16{\cdot}2 \times 3–7\mu$. This flagellate has 3 anterior flagella of unequal length. The posterior flagellum, which trails behind, takes its origin in a separate blepharoplast and is about twice the size of an anterior flagellum. The flagellate has an ellipsoidal nucleus, which in specimens stained by Heidenhain's method shows a central karyosome (Fig. 9). The mouth consists of a narrow slit lying ventrally at the anterior end. The cytoplasm is vacuolated; some of the vacuoles are definitely for the reception of food particles, while others do not contain any solid material and their function is unknown.

### (*d*) Eutrichomastix *from the grass-snake*

A pure-line strain was started from a single organism. No trace of an undulating membrane could be made out in any individual during 4 weeks' close observation. The shape of the body is more or less elongated. Some specimens have their anterior end pointed like a beak. The size varies from 8–14·2 × 4–7 $\mu$. There are 4 flagella—the 3 anterior shorter than the fourth, and usually directed forward in a single bunch. The fourth flagellum is a trailing one but does not differ in thickness and in its staining character from the others. It is nearly twice as long as an anterior flagellum. The axostyle is a hyaline slender rod, usually straight or slightly bent, but in the rounded forms it is very much curved (Figs. 10 and 11). Its posterior end often projects for a short distance beyond the cytoplasm. The nucleus is oval measuring approximately 1·7 × 2·5 $\mu$.

### (*e*) Eutrichomastix *from* Chamaeleon quilensis

There is no apparent morphological difference between this flagellate and the *Eutrichomastix* from snakes. The culture was examined 32 times during a period of 2½ months, and the flagellates always remained true to type.

## IV. Summary and conclusions

Three pure-line strains started from single individuals, 2 of *Trichomonas* and 1 of *Eutrichomastix*, all from snakes, were isolated from mixed cultures. These strains, and another of *Eutrichomastix* from a chamaeleon, were under observation for periods of from 3 to 10 weeks.

The *Eutrichomastix* of snakes does not differ from that of the chamaeleon in any essential point, and neither developed an undulating membrane at any time during this prolonged period of observation.

The *Trichomonas* of the grass-snake is morphologically distinct from that of the boa. *Trichomonas* of the boa has an ill-developed undulating membrane with only a few shallow waves, and may simulate a *Eutrichomastix* owing to the accidental detachment of the bordering flagellum.

*Trichomonas* from snakes, as from most other hosts, readily feeds upon starch granules, whereas none of the *Eutrichomastix* were found to ingest unaltered starch.

*Eutrichomastix* from snakes and the chamaeleon never develops an undulating membrane and does not assume a *Trichomonas* form.

From these observations it can be definitely concluded that *Trichomonas* and *Eutrichomastix* are generically distinct.

I am deeply indebted to Prof. J. G. Thomson for his very kind interest in this work and for the gift of some beautifully stained specimens of the caecal contents of a krait showing a heavy infection with *Eutrichomastix*.

I also wish to record my thanks to Prof. E. Reichenow of the Institut für Schiffs- und Tropenkrankheiten for giving me a culture of trichomonads from *Boa constrictor*.

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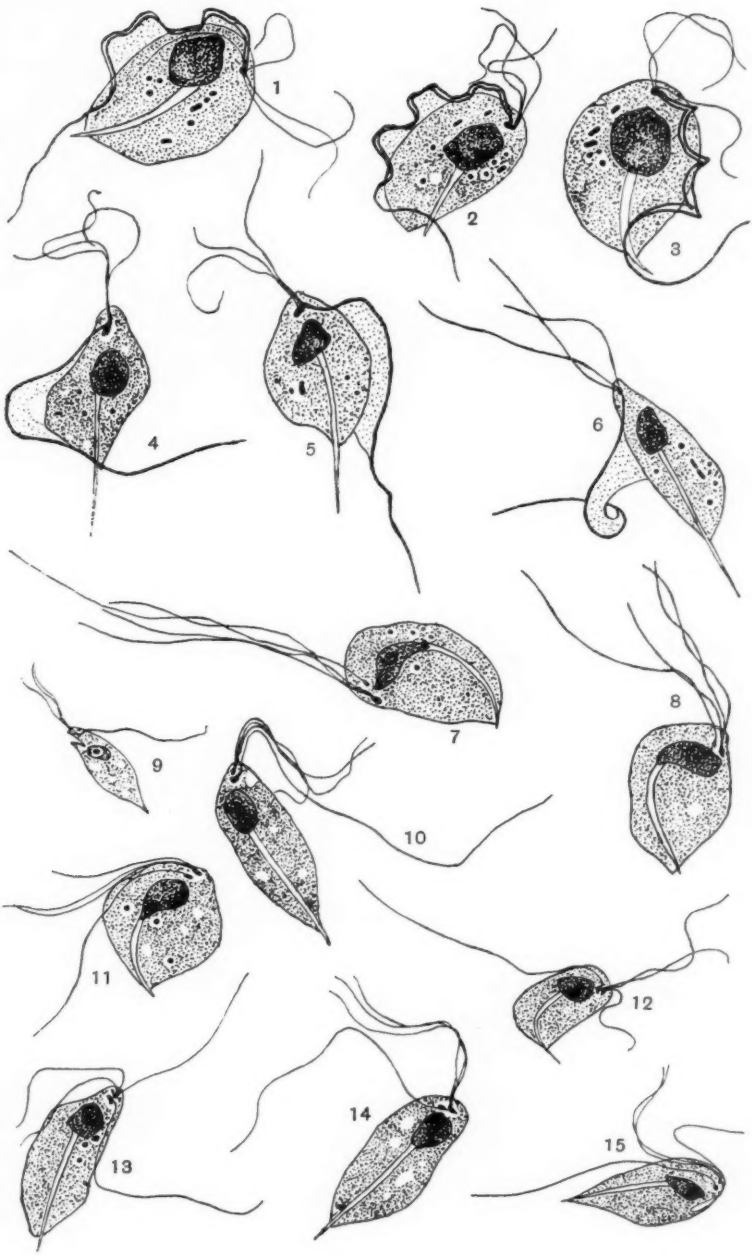
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## EXPLANATION OF PLATE VII

These drawings have been outlined with the aid of the camera lucida at a magnification of about 2000 diameters. All figures are (unless otherwise stated) from slides stained with Giemsa's stain.

Figs. 1–3. *Trichomonas* of the grass-snake, *Natrix natrix*. The undulating membrane has a double marginal flagellum.

Figs. 4–6. *Trichomonas* from *Boa constrictor*. The axostyle is slender and protrudes for some distance beyond the cytoplasm. There is hardly any "wave" in the undulating membrane. Fig. 6 shows partial detachment of its bordering flagellum.

Figs. 7–9. *Eutrichomastix* from the krait (*Bungarus candidus*). Fig. 9 shows the typical nucleus containing a central karyosome (from a smear fixed in Schaudinn's fluid and stained with Heidenhain's iron-haematoxylin).

Figs. 10–12. *Eutrichomastix* of the grass-snake, *Natrix natrix*. Fig. 10. Flagellate with axostyle almost straight. Fig. 11. Rounded individual with axostyle much curved.

Figs. 13–15. *Eutrichomastix* from *Chamaeleon quilensis*.

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# *TRICHOMONAS* SP. FROM THE GUT CONTENTS OF A CORAIS SNAKE

By B. M. DAS GUPTA

*From the Department of Protozoology, London School of Hygiene and Tropical Medicine*

(With 9 Figures in the Text)

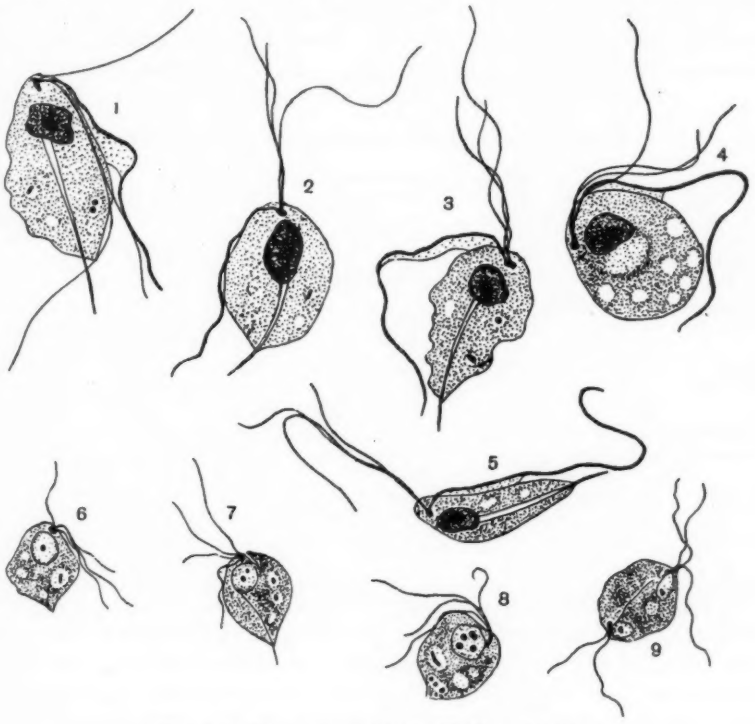
## Material and methods

The material for the present work was made available through the courtesy of Col. A. E. Hamerton. It consisted of the intestinal contents of a corais snake which died at the London Zoological Gardens. A culture was made in serum-saline-citrate solution (Tanabe, 1925) and incubated at 23° C., and a growth of *Trichomonas* was obtained on the fourth day. The strain has since been maintained by subinoculation into haemoglobin-saline medium (Row, 1914) every seventh day. In this medium there was excellent growth on the third day, when smears were prepared and stained with Giemsa's stain by the method previously described (Das Gupta, 1935), and also with Heidenhain's iron-haematoxylin. The haematoxylin method did not prove so satisfactory for the demonstration of the flagella and the undulating membrane, although it was preferable for the study of the nuclear structures, especially during the process of division.

## Morphology

The shape of the body is more or less piriform, although both rounded and slender forms (Figs. 4 and 5) are not uncommon. The length varied from 6 to 18$\mu$, and the width from 3 to 15$\mu$. Most individuals, however, were between 8 and 14$\mu$ long and from 4 to 10$\mu$ broad; the average size would thus appear to be about 12 by 8$\mu$. The measurements were based on 200 unselected flagellates from smears prepared from cultures and stained with Giemsa's stain. The cytoplasm is loaded with food inclusions. There are 3 unequal anterior flagella, the longest being about one and a half times the length of the shortest. The oval nucleus is vesicular with a small central karyosome (Fig. 6), and measures about 3·5 by 2$\mu$. Occasionally two karyosomes may be present (Fig. 7); and during the process of division the karyosome is replaced by 4 or 5 granules (Fig. 8) which possibly represent chromosomes. In the later stages the blepharoplast divides but the connection between the daughter blepharoplasts is maintained by a centrodesmose which persists till almost the end of division (Fig. 9). The undulating membrane is feebly de

veloped, having as a rule only one or two shallow "waves", while in some organisms the membrane shows no undulation at all and simply follows the curve of the body (Fig. 2). Occasionally the flagellum bordering the undu-




Figs. 1–5. From smears stained with Giemsa's stain.

Fig. 1. Normal individual.

Fig. 2. Individual with no definite "waves" in the undulating membrane.

Fig. 3. The flagellum bordering the undulating membrane has been detached and appears like a free flagellum.

Fig. 4. Rounded individual.

Fig. 5. Slender form.

Figs. 6–9. From smears stained with Heidenhain's iron haematoxylin.

Fig. 6. *Trichomonas* in resting stage; nucleus contains a central karyosome.

Fig. 7. Nucleus with 2 karyosomes.

Fig. 8. Nucleus with 4 granules (? chromosomes).

Fig. 9. Late telophase, centrodesmose still present.

(All figures were drawn with the aid of a camera lucida, ×2000.)

lating membrane becomes detached and appears free (Fig. 3). No basal fibre has been seen in any of the many specimens examined. The axostyle is a slender structure and protrudes beyond the body for a distance of 4·5$\mu$ or even more. The cytostome lies close to the blepharoplast (Fig. 7).

## Discussion

Probably the first record of the occurrence of *Trichomonas* in snakes was that of Plimmer (1912) who, during the years 1908–11, found the flagellates in blood smears taken from the animals post-mortem[1]. It may be assumed, however, that although present in the blood they were actually intestinal parasites. Plimmer did not describe fully or name any of these flagellates.

Table I. *List of* Trichomonas *recorded from snakes*

| Snake | Size in microns | Anterior flagella | Undulating membrane | Basal fibre | Locality | Observer and date |
|---|---|---|---|---|---|---|
| *Coluber leopardinus* | — | — | — | — | Europe | Plimmer (1912) |
| *Naia tripudians* | — | — | — | — | India | ,, |
| *Heterodon simus* | — | — | — | — | N. America | ,, |
| *Python sebae* | — | — | — | — | W. Africa | ,, |
| *P. molurus* | — | 3 | — | — | India | Wenyon (1926) |
| *Ablabes calmaria* | — | 4 | 3 or 4 deep waves | Present | ,, | Das Gupta (1927) |
| *Boa constrictor* | — | 3 | 1 or 2 shallow waves | Absent | — | Reichenow (1928) |
| *Natrix erythrogaster* | 12·8$\mu$ av. length | 4 | 3 or more deep waves | Present | U.S.A. | Das Gupta (1935) |
| *N. natrix* | 11–17·5 by 4–12 | 3 | 3 or more deep waves (lat. flagellum has two filaments) | Absent | England | ,, |

From the insufficient data available it is difficult to differentiate with certainty the species of *Trichomonas* so far observed in snakes. It will be seen from Table I that the *Trichomonas* from *Ablabes calmaria* and *Natrix erythrogaster* have 4 anterior flagella, a well-developed undulating membrane, and a distinct basal fibre. The *Trichomonas* from *Boa constrictor* and *Natrix natrix* have 3 free flagella and no basal fibre, but the former differs from the latter in that it has a well-developed undulating membrane, and the flagellum forming the outer margin of the membrane has two distinct filaments. It thus appears that up to the present at least 3 distinct species of *Trichomonas* have been described in snakes. The present species bears a striking resemblance to the *Trichomonas* from *Boa constrictor*. Like the latter it possesses a poorly developed undulating membrane, while in both the flagellum bordering this membrane often becomes detached probably as a result of slight pressure in preparing the smear. Such forms may be confused with *Eutrichomastix*, a flagellate which is often present in the intestine of many reptiles. It seems quite probable that the *Trichomonas* from the corais snake is of the same species as that from *Boa constrictor*, viz. *Trichomonas boae* Reichenow, 1928.

[1] [Dobell, however, observed a *Trichomonas* in the intestine of a green whip-snake (*Dryophis mycterizans*) in Ceylon, in 1909, and reported this in the following year. See *Spolia zeylan.* (1910), **7**, 73.]

SUMMARY

Trichomonas *sp. from a corais snake*

*Description.* Body usually pear-shaped. Average size $12 \times 8\mu$. Three anterior flagella of unequal length. Basal fibre absent. Feebly developed undulating membrane.

*Habitat.* Intestine of *Dymarchon corais*, from North America.

*Locality.* London.

I wish to express my gratitude to Prof. J. G. Thomson for providing all the facilities for carrying out this work.

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# ON *EUTRICHOMASTIX* OF SOME AFRICAN SNAKES

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(With 4 Figures in the Text)

## Material and methods

Dr C. A. Hoare, Protozoologist to the Wellcome Bureau of Scientific Research, very kindly provided smears of the intestinal contents of 7 species of snakes which he had collected while in Uganda. Of these, 3 different species, viz. *Psammophis sibilans*, *Boaedon lineatus* and *Crotaphopeltis hotamboeia*, were infected with *Eutrichomastix*. The smears were fixed in Schaudinn's fluid and

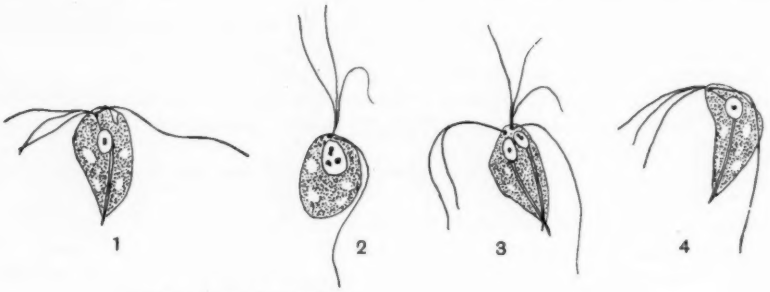


Fig. 1. Normal individual.
Fig. 2. Individual with the nucleus containing 4 granules.
Fig. 3. "Double individual."
Fig. 4. Form showing a beak-like protuberance.

All figures were drawn with a camera lucida from preparations fixed in Schaudinn's fluid and stained with iron-haematoxylin. Magnification *ca.* 2000 diameters.

stained with iron-haematoxylin. The flagellates from all snakes were morphologically identical. This study was based mainly on the parasites found in the last-named host, which showed a heavy infection.

## Description

The shape is usually piriform. Some, however, have elongated or rounded bodies. The flagellate measures 4–10 × 2–5 $\mu$. A few individuals show a beak-like projection at the anterior end (Fig. 4). There are 3 anterior flagella of almost equal length, and the trailing posterior flagellum is much longer. The axostyle is slender and slightly curved. There is a small cytostome close to

the blepharoplast (Fig. 1). The blepharoplast is connected with the nucleus by a rhizoplast (Figs. 1 and 4). The nucleus is spheroidal and has a central karyosome. Flagellates (Fig. 2) with the nucleus preparing to divide have been seen. In some individuals (Fig. 3) various organellae are duplicated. These forms are probably similar to the "double individuals" seen in cultures of *Trichomonas hominis* by Bishop (1931). In all essential features this flagellate appears to be identical with *Eutrichomastix serpentis* (Dobell, 1907) Kofoid & Swezy, 1915. The size of *E. serpentis* is given as 8–17 $\mu$ long, whereas the flagellate described here measures only 4–10 $\mu$ in length. This discrepancy can be explained by the fact that the specimens on which the present study is based were fixed in Schaudinn's fluid, which invariably causes shrinkage of the organism; whereas, in the case of *E. serpentis*, the measurements were apparently taken from fresh preparations.

Table I. *Snakes from which* Eutrichomastix *has previously been recorded*

| Host | Observer and date |
|---|---|
| *Vipera aspis* | Léger (1904) |
| *Boa constrictor* | Dobell (1907) |
| *Dryophis mycterizans* | Dobell (1910) |
| *Pituophis catenifer*, *Eutaenia sirtalis*, *Crotalus oregonus*, *Python reticulatus* | Kofoid & Swezy (1915) |

SUMMARY

A *Eutrichomastix* morphologically identical with *E. serpentis* (Dobell, 1907) Kofoid & Swezy, 1915, is described from 3 species of African snakes, namely, *Psammophis sibilans*, *Boaedon lineatus* and *Crotaphopeltis hotamboeia*.

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# MORPHOLOGY AND LIFE-CYCLE OF AN AMOEBA PRODUCING AMOEBIASIS IN REPTILES[1]

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(With Plates VIII and IX, containing Figs. 1–39 and Fig. I in the Text)

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## Introduction

Pathogenic species of Protozoa are greatly outnumbered by non-pathogenic ones. Parasitic species of a genus may be closely related morphologically, yet one may produce disease, whereas the others may be harmless. In the genus *Entamoeba* Casagrandi et Barbagallo 1895, this fact is well demonstrated, since species have been described as parasites in practically all phyla of the animal kingdom, including the recent discovery (Stabler, 1933; Carini & Reichenow, 1935) of a hyperparasitic *Entamoeba* in *Zelleriella*, an astomatous ciliate of Amphibia. Of all the known species of *Entamoeba*, the dysentery amoeba of man and other mammals, *E. histolytica*, was the only known pathogenic form prior to 1933[3]. In a number of the descriptions, similarities in movement and morphology to *E. histolytica* were noted, but no disease was described.

[1] This work was started at the Laboratory of Comparative Pathology, Zoological Gardens, Philadelphia, Pennsylvania, and completed at the Department of Comparative Pathology, Harvard Medical School, Boston. Acknowledgment is made to Research Grant No. 172 of the University of Pennsylvania Faculty Research Fund for support in obtaining materials and supplies.

[2] Fellow of the National Research Council.

[3] In 1922, Ilowaisky (*Arch. russes Protist.* **1**, 82) described amoebic hepatitis of frogs, *Rana temporaria*, but did not present sufficient pathological evidence to establish the disease without question.

However, in 1933 and 1934 Ratcliffe and Geiman described a disease in certain reptiles which was comparable in its essential features to amoebiasis in man and which was directly associated with an *Entamoeba* sp. strikingly similar in morphology to *E. histolytica*. No specific name was given to the amoeba on those two occasions because of its similarities to *E. histolytica* and to several species of amoebae already described from reptiles and because it was found necessary to make a thorough morphological and physiological comparison with each of the described species. Therefore, the morphology and life cycle of this *Entamoeba* and a morphological comparison with previously described species from related hosts is presented below.

Late in 1934, J. Rodhain described "*Entamoeba invadens* n.sp., parasite de serpents" and in collaboration with M. Th. van Hoof published a "Note préliminaire sur le rôle pathogène possible d'*Entamoeba invadens* chez les serpents." This organism may be closely related to, or even identical with the one originally discovered by us in 1933, but the description of the *Entamoeba* is too inadequate to serve as a reliable morphological basis for its identification as a distinct species. However, in view of the pathogenicity of Rodhain's *Entamoeba* and of its possible identity with the species which we have already reported, it appears preferable for the present to assign the organism which we describe below to his species, *E. invadens*, rather than create a new one. If, later on, more complete information about his species should show it to be distinct from the amoeba with which we are working, a new species may then be created for the latter.

Acknowledgments are made to Dr E. E. Tyzzer and other members of the Department of Comparative Pathology, and to Dr L. R. Cleveland of the Department of Tropical Medicine, Harvard Medical School, for their advice and criticism and also for facilities offered during the final stages of the research. The suggestions and advice of Dr D. H. Wenrich, University of Pennsylvania, were also very helpful during the early stages of the work.

## MATERIAL AND METHODS

The behaviour and appearance in life of the organism associated with amoebic disease in certain reptiles was studied from saline preparations made directly from the hosts and from cultures. Permanent smear preparations were fixed with Schaudinn's fixative plus 5 per cent. of glacial acetic acid, half strength Schaudinn's fixative plus 2 per cent. of glacial acetic acid, and Bouin's fluid (Allen's P.F.A.$_3$ (C. E. McClung, 1929, *A Handbook of Microscopical Technique*, New York, p. 424) and Grassé's (P. P. Grassé, 1926, Contribution à l'étude des Flagellés Parasites, *Arch. Zool. Exper. et Gén.*, **65**, p. 349) modifications.) Tissues for sectioning were fixed with Zenker's fluid and Bouin's fluid. Heidenhain's iron-alum-haematoxylin, Mayer's haemalum and eosin, and the eosin methylene-blue method of Mallory were used for staining. Smear preparations and sections were made from intestines, liver, and stomach of infected hosts. Cultures were also the source of many

preparations. The hosts from which the preparations were derived included: 26 water snakes (14 *Natrix. rhombifer*, 3 *N. sipedon*, 6 *N. sipedon* var. *sipedon*, and 3 *N. cyclopion*), 2 black snakes (*Coluber constrictor*), and 2 monitors (1 *Varanus salvator* and 1 *V. varius*). All snakes dying of spontaneous disease or used for experimental infection are native in the southern and eastern United States. The two species of *Varanus* were obtained for the Zoological Society of Philadelphia from an animal dealer in New York City, but they could not be traced to their natural source. It should be noted that other species of reptiles (listed under species diagnosis) died of amoebiasis due apparently to the same organism, but, for one reason or another, the amoebae from these additional hosts were either present only in small numbers after autopsy, or smears proved unsatisfactory for cytological study.

Since the beginning of the work, 18 strains of this reptile *Entamoeba* have been cultivated for periods varying from a few weeks up to 22 months at the date of writing. The culture media used have already been described elsewhere (Ratcliffe & Geiman, 1934 *a*). The most satisfactory medium of that series was 9 parts of 0·5 per cent. saline and 1 part of horse serum plus rice starch, a modification of the medium used by Barret & Smith (1924) to cultivate *E. barreti*. The use of that medium was discontinued about a year ago in favour of the gastric mucin-saline medium of Ratcliffe (1934) plus rice starch. This later medium (0·3 per cent. gastric mucin in 0·5 per cent. saline) has proved more satisfactory for constant cyst production. Maximum culture growth is maintained at room temperature (20–30° C.) by subculture every 7 days.

Excystation and metacystic development have been followed by feeding to snakes cysts from two prolific strains, designated strains *A* and *B*, secured respectively from a *Natrix rhombifer* dying of amoebic disease and from a *Varanus varius* dying of a similar disease. Two pure lines were obtained from cultures of strain *A* by the isolation of single trophozoites. These two pure lines were used to supplement the original cultures for studies on excystation and metacystic development and also to determine whether or not the original strain *A* was composed of one or several strains.

## Morphology and size

### A. *Morphology*

#### (*a*) *Trophozoites.*

Observations of saline preparations direct from the diseased tissues or from 2- to 7-day cultures reveal an actively motile amoeba. When such preparations are made and sealed quickly so that exposure to oxygen is reduced, there may be observed the explosive extrusion of clear, ectoplasmic pseudopodia upon change of direction. However, when the amoeba is moving continuously in one direction no ectoplasmic layer can be distinguished, but occasionally a tuft of débris can be seen adhering to the posterior end. The amoebae move

forward quite rapidly in a slug-like manner with typical amoeboid movement and obvious streaming of the food vacuoles and the nucleus in the endoplasm. The nucleus is readily seen in young trophozoites containing no rice starch or other food particles, and maintains its shape in young organisms during locomotion. The hyaline refractive peripheral chromatin of the nucleus may be seen, and at times the karyosome is faintly visible in the centre. The nuclei in amoebae from old cultures are elastic, and occasionally the chromatin tends to degenerate and clump in masses. However, the nuclear structure of *Entamoeba invadens* is less variable and more constant under changing temperature and cultural conditions than in any other *Entamoeba* from Amphibia, reptiles, and mammals studied by the writers. This greater stability of the nucleus may be explained on the basis of adaptability of the amoeba to various culture media and also to survival at a temperature range of 10–33° C. Of course this adaptability may also explain the ability of this amoeba to infect various reptilian hosts.

In stained preparations, the endoplasm of the trophozoites appears dense and contains the food vacuoles and the nucleus (Pl. VIII, Fig. 1). The food vacuoles may contain host-cell débris, liver cells and leucocytes in process of digestion, and bacteria (Figs. 1 and 3). No erythrocytes of the host have been definitely identified at any time in the parasites, nor have we ever been able to demonstrate the ingestion of human or horse red cells. The amoebae, on the other hand, will begin to ingest grains of rice starch within a few seconds after they are added to cultures or fresh preparations. The starch grains are taken up until the whole body is engorged and rounded.

The clear ectoplasmic pseudopodia are rounded and their typical position after their explosive formation is shown in Fig. 1. These pseudopodia rarely become slender and arm-like, in fact such pseudopodia have been noted only twice in cultures several weeks after transfer.

The nucleus is vesicular and contains all the structures typical of *Entamoeba*. There is a very thin nuclear membrane. Discrete, heavily staining chromatin granules lie directly underneath and are often packed closely against the nuclear membrane (Fig. 2). The karyosome may be seen as a distinct central granule (Figs. 5 and 6), but more often appears to consist of several granules (Fig. 1) as described for *E. histolytica* by Dobell (1928). A clear halo surrounds the karyosome which in turn is surrounded by an "achromatic cloud". Occasionally "spoke radii" can be detected connecting the karyosome and the peripheral chromatin (Fig. 3).

(*b*) *Cysts.*

*Entamoeba invadens* produces quadrinucleate cysts (Figs. 10–18). Cysts in all stages of development have been obtained from lesions in the colon, duodenum, stomach, and liver. While no great numbers of live cysts have been obtained from the lumen of the stomach and intestines of snakes at autopsy, the diseased liver and sometimes the diseased tissue of the colon of

snakes dying of spontaneous disease usually contain early stages of encystation as well as many quadrinucleate cysts. The development of cysts in the diseased liver varies directly with the period between the death of the snake and the time of autopsy. No cysts have been found in the diseased livers of experimentally infected snakes when they were killed and examined immediately. The liver in such cases may be completely involved and contain many trophozoites, but no cysts have ever been found. However, cysts have been produced in large numbers by placing part of a diseased liver under anaerobic conditions for 24 hours. Adequate controls kept under aerobic conditions at room temperature and in a refrigerator failed to produce cysts, although trophozoites were present in large numbers at the end of the 24-hour period.

When cultures are inoculated with amoebae from a snake, cyst production usually begins within 24 hours. The development of cysts in culture as revealed by stained preparations, is in every way comparable to cyst production, as it takes place in the intestine or liver of the host after death.

The hyaline cysts are generally spherical and are easily identified in fresh preparations from the intestine, liver, or cultures. With the exceptions that the cysts are seldom asymmetrical and that the cyst wall is thicker, they are similar to those of *E. histolytica*.

Iodine preparations, containing both trophozoites and cysts, show all the characteristics of *E. histolytica*, so that a repetition of description in detail is considered unnecessary. The characteristics of nuclei in trophozoites and cysts, and of glycogen vacuoles, both in precystic individuals and in cysts, are apparently identical with those of *E. histolytica*.

In stained preparations of cysts, the glycogen vacuole is often large enough to press the nucleus and chromatoidal substance tightly against the cyst wall (Fig. 11). As the development of the cyst proceeds, the vacuole becomes smaller until it disappears at maturity (Fig. 18).

The shape, size, and amount of chromatoidal substance in the cysts of some species of amoebae are considered to be of diagnostic value. In the cysts of *E. invadens*, from the host or from cultures, the chromatoids vary in both shape and size. There are variations from cylindrical rods, sometimes in pairs (Figs. 11 and 13), to irregular masses (Fig. 14) and occasionally acicular bodies. The amount of chromatoidal substance seems to vary also according to the place in which the cysts have developed. For example, cysts formed in the liver usually contain more and smaller chromatoids than those formed in the intestine. Consequently, the variation of chromatoids in this snake amoeba is quite as great as in *E. histolytica*.

### B. *Size*

Measurements of trophozoites and cysts of *E. invadens* are presented in Tables I and II. All measurements were taken at random from fixed and stained preparations made at autopsy from 5 *Natrix rhombifer*, 2 *N. sipedon*, and 4 experimental infections including 1 *N. rhombifer* and 1 *Coluber constrictor*

infected with strain *B* and 1 *Natrix sipedon* var. *sipedon* and 1 *Coluber constrictor* infected with strain *A*. No measurements were made from culture strains because of the increased size resulting from ingestion of rice starch.

(*a*) *Trophozoites.*

A comparison is shown in Table I of the size range of trophozoites from given organs of different individuals and species as well as in two instances from the intestines and liver of the same individual. This table was compiled to illustrate the variations in size and the ratios of average nuclear diameter to average trophozoite diameter encountered; and to illustrate the need for a large series of measurements in order to arrive at a reliable size range for specific diagnosis.

The organisms from each snake are of the same species, as evidenced by their morphology and the production of an identical disease in all these hosts. The relatively high nucleo-cytoplasmic ratio of intestinal amoebae of Nos. 248, 254 and 256 and the lower nucleo-cytoplasmic ratio in the liver of No. 270 are the results of the physiological environmental effects, rapid multiplication, and the presence of precystic amoebae at the time of fixation. The increase in the size of trophozoites in culture has already been mentioned, but it is interesting to note that the organisms retained some of this increase in size during the production of disease by experimental infection (see Nos. 333, 349 and 351). Since the nucleus increased proportionately in diameter, the ratio remained about the same. However, the causes for the maintenance of increased cell size upon experimental inoculation from culture into the original hosts are not understood. It may be that cell size begins to decrease after the host dies of spontaneous disease while the amoebae in the 3 experimental snakes (Nos. 333, 349 and 351) examined immediately after they were killed had no opportunity to decrease in size.

(*b*) *Cysts.*

The size range of the cysts presented in Table II also shows an increase in average size in experimentally infected animals. These measurements were all made from liver smears for comparison. In the cases containing cysts in intestinal smears, measurements agree closely with those found in the liver.

Table II. *Measurements of stained cysts of* Entamoeba invadens *from spontaneous and experimental amoebic liver infections*

| No. | Host species | Spontaneous or experimental | No. measured | Range of size in $\mu$ | Av. diam. in $\mu$ |
|---|---|---|---|---|---|
| 254 | *Natrix rhombifer* | S | 100 | 11·0–19·0 | 13·06 |
| 256 | ,, | S | 50 | 11·0–15·5 | 13·2 |
| 333 | ,, | E (strain *B*) | 50 | 11·0–20·2 | 14·2 |
| 350 | *Coluber constrictor* | E (strain *A*) | 50 | 13·8–20·2 | 15·9 |
| | | | Total | Range | Av. |
| | | | 250 | 11·0–20·2 | 13·88 |

Table I. *Measurements of stained trophozoites of* Entamoeba invadens *from the diseased intestines and livers of snakes*

| | | | Measurements from the intestine | | | | | | | Measurements from the liver | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Trophozoite | | | Nucleus | | Ratio Nuclear diam./ Trophozoite diam. | | Trophozoite | | | Nucleus | | Ratio Nuclear diam./ Trophozoite diam. |
| No. | Host species | Nature of infection* | No. measured | Range of size μ | Av. length | Av. width | Range of size μ | Av. diam. | | No. measured | Range of size μ | Av. length | Av. width | Range of size μ | Av. diam. | |
| 248 | *Natrix rhombifer* | S | 50 | 11–21 × 8–17 | 14·4 | 12·7 | 3·5–5·5 | 4·52 | 0·33 | — | — | — | — | — | — | — |
| 254 | ,, | S | 50 | 10–20 × 8–18 | 13·5 | 11·4 | 3·5–6·0 | 4·24 | 0·34 | 50 | 11–24 × 10–22 | 17·5 | 15·1 | 3·6–5·5 | 4·9 | 0·30 |
| 256 | ,, | S | 50 | 12–22 × 10–18 | 15·6 | 13·4 | 3·5–7·0 | 4·68 | 0·32 | 50 | 13–24 × 11–22 | 17·8 | 15·2 | 3·6–6·0 | 4·8 | 0·29 |
| 269 | ,, | S | — | — | — | — | — | — | — | 25 | 14–21 × 12–20 | 16·7 | 15·0 | 4·0–5·5 | 4·7 | 0·29 |
| 270 | ,, | S | — | — | — | — | — | — | — | 50 | 14–26 × 11–23 | 19·0 | 17·1 | 3·6–5·5 | 4·7 | 0·26 |
| 298 | *Natrix sipedon* | S | 25 | 12–21 × 9–18 | 16·4 | 12·9 | 3·5–5·5 | 4·5 | 0·30 | — | — | — | — | — | — | — |
| 299 | ,, | S | 25 | 11–23 × 11–18·4 | 16·0 | 13·3 | 3·6–5·5 | 4·2 | 0·28 | — | — | — | — | — | — | — |
| 333 | *Natrix rhombifer* | E (*B*) | — | — | — | — | — | — | — | 50 | 9·2–31 × 9·2–22 | 19·0 | 15·0 | 4·0–6·4 | 5·0 | 0·29 |
| 349 | *Natrix sipedon sipedon* | E (*A*) | 50 | 11–34 × 11–30 | 18·2 | 16·0 | 3·6–7·3 | 5·0 | 0·29 | — | — | — | — | — | — | — |
| 351 | *Coluber constrictor* | E (*B*) | — | — | — | — | — | — | — | 25 | 15·6–38·6 × 11·9–27·6 | 22·8 | 19·1 | 4·6–7·3 | 6·2 | 0·30 |
| | | | Total | | Av. | Av. | | Av. | Av. | Total | | Av. | Av. | | Av. | Av. |
| | | | 250 | | 15·68 | 13·29 | | 4·57 | 0·31 | 250 | | 18·8 | 16·0 | | 5·0 | 0·28 |

* S = spontaneous infection, and E = experimental infection.

## ENCYSTATION AND EXCYSTATION

### A. *Encystation*

The precystic amoebae (Fig. 9) are usually rounded and much smaller than the normal trophozoites, and the nucleus is frequently enlarged in proportion to the diameter of the individual. Sometimes the beginning of chromatoid formation can be seen around the glycogen as the hyaline cyst wall is being secreted. The chromatoids begin to form as many individual, irregular, deeply staining bodies in the cytoplasm. By the time that the cyst wall is secreted, a very large glycogen mass is well defined, pushing the now definitely shaped chromatoids and nucleus against the cyst wall. Division of the nucleus appears to be initiated under certain conditions very soon after the cyst is formed (Fig. 12). The nucleus of a newly formed cyst divides once (Fig. 12) to form two nuclei (Figs. 13 and 14), and then each of these divides again to form the typical quadrinucleate cyst (Fig. 17). By this time, the chromatoidal substance and the glycogen have decreased in amount from that usually found in a uninucleate cyst. However, when the quadrinucleate cysts are stored in a refrigerator (3–10° C.) for a week or 10 days, stained preparations show that they mature by the absorption of the glycogen and chromatoidal substance (Fig. 18). When this process is completed, excystation can take place provided the required surroundings obtain.

### B. *Excystation*

Since this disease-producing amoeba grows readily at room temperature and a fairly constant number of cysts is produced every 7 days in the gastric-mucin-saline medium plus rice starch, the various excystation methods of Dobell (1928), Cleveland & Sanders (1930), and Sanders & Cleveland (1930) were tried. Although a certain amount of excystation took place, and metacystic stages have been seen in such culture preparations, mass excystation necessary for the making of slides and study did not occur. Thus before proceeding with further culture experiments, it was believed that excystation and metacystic development could be obtained by inoculating snakes with large numbers of washed mature cysts freed of trophozoites by refrigeration. Until the recent work of Tanabe (1934), excystation and metacystic development of an *Entamoeba* as it takes place in the host had never been worked out in detail. Chatton (1917) fed cysts of *E. histolytica* to a number of cats, and after a series of observations concluded that the cyst wall was digested during passage to free a quadrinucleate amoeba in the intestine. The figures of Chatton are without question of metacystic amoebae resulting from excystation, but he failed to discover the process of metacystic development as later described by Dobell (1928), and Cleveland & Sanders (1930). Hegner, Johnson & Stabler (1932) obtained excystation in monkeys by the injection of *E. histolytica* cysts into the stomach of three juvenile brown howler monkeys, but their figures and description of the process do not agree with those of Dobell (1928).

Three snakes were used in the first experiment to test the proposed method of obtaining excystation and metacystic development. Mature cysts (strain A) were inoculated by mouth with the aid of a steel catheter. Snake No. 1 (*Natrix rhombifer*), killed 1 hour after inoculation, still had the majority of cysts in the stomach. Snake No. 2 (*N. sipedon* var. *sipedon*), killed 3 hours after inoculation, had the inoculum about equally divided between the stomach and duodenum. No movement was seen in any of the cysts. Snake No. 3 (*N. rhombifer*), killed 5 hours after inoculation, had most of the inoculum in the jejunum with a small amount still in the duodenum. Excystation was proceeding actively. Movement could be seen in nearly all cysts not already empty, and a number of metacystic amoebae were observed moving about. Thus the results with snake No. 3 showed that the method was feasible and also that the approximate time necessary for excystation was 5 hours.

Nine additional snakes of 3 species were inoculated and killed at 5, 7, 9, 11, 12, 14, 18 and 24 hours after inoculation. The inoculum usually remained fairly well concentrated while passing down the alimentary tract, so that smears showing the desired stages were easily obtained. Strain *A* was used for all experiments, with the exception of snake No. 5. In this snake, the examination of excystation in fresh preparations and in stained smears showed that the process was similar. The record of the additional 9 snakes inoculated, strain of cysts used, time when killed after inoculation, and position of the majority of cysts and metacystic amoebae is as follows:

No. 4, *N. rhombifer*, strain A, 5 hours, jejunum and ileum.

No. 5, *N. rhombifer*, strain *B*, 7 hours, jejunum.

No. 6, *N. rhombifer*, strain *A*, 9 hours, upper ileum.

No. 7, *N. sipedon sipedon*, strain *A*, 11 hours, stomach (due to worm infection).

No. 8, *N. cyclopion*, strain *A*, 11 hours, upper ileum.

No. 9, *N. cyclopion*, strain *A*, 12 hours, ileum.

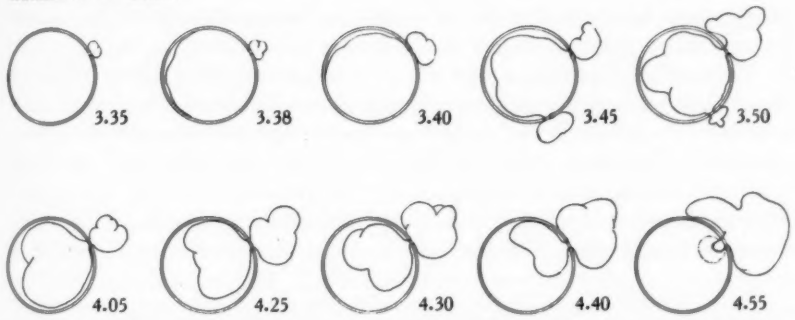
No. 10, *N. cyclopion*, strain *A*, 14 hours, ileum.

No. 11, *N. sipedon sipedon*, strain *A*, 18 hours, ileum.

No. 12, *N. sipedon sipedon*, strain *A*, 24 hours, ileum and colon.

The process of excystation even as to minute details (Figs. 19–23) is entirely in agreement with that described by Dobell (1928) for *Entamoeba histolytica*. Another detailed account at this time would be superfluous. Text-fig. 1 shows free-hand sketches of the process as observed in snake No. 3 over a period of 1 hour and 15 min. Stages at 3.45 and 3.50 show protrusion of protoplasm from two apertures in the cyst wall and this is also shown in Fig. 21 drawn from a stained slide. Although there were two openings in this cyst, the amoeba finally emerged through only one of them. The length of time required in the case illustrated by the text-figure is evidently the maximum necessary for complete emergence. Other cysts on this same slide were seen to excyst in 15 min. from the time when the amoeba was first observed to loosen itself from the cyst wall and move about within the cyst.

During the whole series of experiments excystation was not found in any stained smears beyond 14 hours after inoculation, although in snake No. 10 excysting amoebae were unmistakably found in the stained preparations at the end of this interval. Thus, these experiments indicate that excystation may take place 5–14 hours after inoculation, but the majority of cysts excyst within 5–7 hours.




Text-fig. 1. Diagrams showing the excystation of *Entamoeba invadens* as it was observed to take place in 1 hour and 28 minutes in saline solution. The process began at 3.35 p.m. and the amoeba was free from the cyst wall at 5.03 p.m. Material for observation was obtained from the intestine of a snake inoculated 5 hours previously with mature cysts of *E. invadens*. The stage at 3.45 p.m. shows the protrusion of protoplasm through two openings, similar to the stage found in a stained preparation and figured on Plate IX, Fig. 21.

## METACYSTIC DEVELOPMENT IN THE SNAKE'S INTESTINE

Without exception, each mature amoebic cyst excysts to form a metacystic amoeba with 4 "cystic" nuclei. (See Dobell (1928) for the original terms and schematic representation used to describe the metacystic development for *E. histolytica*.) This metacystic amoeba begins to feed immediately and grows quite rapidly. Its four nuclei are usually clumped in a characteristic manner (Fig. 25). Each cystic or *N* nucleus sooner or later divides to form *nn* nuclei as demonstrated for *E. histolytica* by Dobell (1928) and Cleveland & Sanders (1930). Thus the 4 *N* nuclei of the recently emerged metacystic amoeba ultimately divide once to form 8 *n* nuclei. Since cytoplasmic division and the parcelling out of the cystic and metacystic nuclei is not always equal, amoebae with 24 different combinations of *N* and *n* nuclei are possible in the process of formation of 8 amoebulae from each 4 *N* metacystic amoeba (Cleveland & Sanders, 1930). Cleveland & Sanders (1930) found all these combinations, but while we have failed to find or differentiate the 1 *N*, 4 *n*, 5 *n*, *Nnnnnn*, and *NNnnn* combinations, 19 combinations of nuclei have been identified in our preparations. Amoebae with the smaller numbers of nuclei occurred more frequently than the 6-, 7- and 8-nucleate amoebae.

In Figs. 25–35, we have shown some of the combinations of *N* and *n* nuclei occurring in the metacystic development leading to the formation of 8 amoebulae from each quadrinucleate metacystic amoeba. Two quadrinucleate (4 *N*)

metacystic amoebae (Figs. 24 and 25) showing ingested bacteria are figured. The division of a 1 *N* nucleus to form a *NNNnn* combination is represented in Fig. 26. The 4 cystic nuclei may divide to form an 8 *n* metacystic amoeba (Fig. 33), and Fig. 32 shows a *Nnnnnnn* combination. However, metacystic amoebae with 6–8 nuclei were not so common, and the frequency of the smaller numbers of *Nn* combinations (Figs. 28–31 and 34–35) found in our preparations was probably due to conditions favourable for the division of the cytoplasm as the nuclei divided.

We have attempted to analyse by random counting the relative incidence of 4 *N* metacystic amoebae, metacystic amoebae with combinations of *N* and *n* nuclei or more than 1 *n* nucleus, and amoebulae with a 1 *n* nucleus at various times after inoculation. Table III shows the results, and indicates for the most part that the metacystic development is completed at the end of 24 hours after the ingestion of mature cysts. After 7 hours, the formation of *Nn* combinations begins along with the formation of a few amoebulae, but most of

Table III. *Relative incidence of three different kinds of metacystic amoebae during the development of* Entamoeba invadens

| | | Metacystic amoebae | | | |
|---|---|---|---|---|---|
| No. of snake | Time after inoculation hours | No. with 4 *N* nuclei | No. with *Nn* or *nn* nuclei | No. with 1 *n* nucleus | Total counted |
| 3 | 5 | 92 | 6 | 2 | 100 |
| 5 | 7 | 76 | 17 | 7 | 100 |
| 9 | 12 | 115 | 87 | 48 | 250 |
| 10 | 14 | 66 | 17 | 17 | 100 |
| 11 | 18 | 124 | 37 | 89 | 250 |
| 12 | 24 | 9 | 34 | 207 | 250 |

the development takes place during the second 12-hour period after inoculation. After 18 hours, there are almost twice as many amoebulae as after 12 hours, and after 24 hours there are approximately 5 times as many uninucleate amoebae as after 12 hours. It is to be expected that the incidence of these types of amoebae may vary in snakes killed at similar intervals after inoculation. Thus there are actually fewer metacystic combinations of *Nn* amoebae in snake No. 10 killed at 14 hours than in snake No. 9 killed after 12 hours. This variation we interpret as the result of inoculation of cysts not quite mature, or of the complication of worm infections in the stomach—which occur in variable degrees. In fact, the inoculum tends to be retained for an indefinite period in the stomach in the presence of a heavy worm infection, as in snake No. 7.

### Multiple fission without encystment

Varying numbers of trophozoites containing chromatoids (Fig. 5) have been noted frequently in our stained preparations made from cultures (particularly horse-serum-saline) containing cysts and typical trophozoites. These trophozoites were usually above the average size, and sometimes the cytoplasm

was literally packed with chromatoidal substance. The chromatoids varied greatly in shape and size, being cylindrical, sometimes in pairs, and occasionally splinter-like.

Cleveland & Sanders (1930) described the process of multiple fission without encystment for *E. histolytica* in cultures. They figured trophozoites containing chromatoids and also showed the division of the nuclei and the formation of quadrinucleate amoebae. However, the fate of these quadrinucleate chromatoid-bearing amoebae was not determined because of the presence of 4 *N* metacystic amoebae in the cultures at the same time, but they did show that the amoebae were not derived from cysts bearing chromatoids.

In the liver-infusion-agar medium of Cleveland & Sanders, all stages in the life cycle of *E. histolytica* can occur simultaneously, but our repeated efforts to demonstrate the life cycle of *E. invadens* as a continuous process in our media have been unsuccessful. However, chromatoid-bearing trophozoites with two nuclei of the same size and also with a large and a small nucleus (Fig. 6) have been found. The significance of the latter type, usually giant organisms, has not been determined. Since no quadrinucleate amoebae have been found in these preparations to suggest the equivalent of cyst formation and metacystic development in the typical life cycle, the fate of trophozoites containing chromatoids remains unknown.

### CANNIBALISM

The phenomenon of cyst ingestion by trophozoites (Fig. 7) is quite common with this amoeba in cultures in which the starch grains are depleted. Sometimes some of the larger trophozoites ingest two or three cysts. Whether the cysts are actually utilised for food has not been determined. However, the contents of the ingested cyst usually begin to degenerate shortly after it has been engulfed by a trophozoite. Cleveland & Sanders (1930) have described this phenomenon of cannibalism of cysts for *E. histolytica* in cultures.

### RELATION TO DESCRIBED SPECIES OF *ENTAMOEBA*

In an attempt to establish the relationship of the amoeba described here, a comparison with other described species of *Entamoeba* from reptiles and certain other vertebrates must be made. The task is not simple because the genus *Entamoeba* contains a heterogeneous group of amoebae. Morphological differences between many species are vague, and many have been named only because of their occurrence in a certain host. Cystic stages have not always been described when a so-called new species was created; but when the mature cysts have been found, they contain either 4 or 8 nuclei. The variability of specific characters in the trophic stages makes necessary the study of the cyst structure for reliable diagnostic identification of species. However, when both trophic and cystic stages are morphologically similar for strains of *Entamoeba* that occur in two distantly related hosts, physiological characters for species differentiation have to be used if differentiation is to be made. This problem of

morphological *versus* physiological characters is involved in trying to establish the relationships of *E. invadens* to other *Entamoebae*. The need for more detailed knowledge concerning the life cycles of a larger series of species of *Entamoeba* is to be emphasised.

*E. testudinis* Hartmann 1910, was the first amoeba to be described from reptiles, but without reference to cystic stages. The host was a turtle, *Testudo graeca*, and the size given for the amoeba was 50–70 $\mu$, nucleus 11·5–12·5 $\mu$. The size and nuclear structure, however, serve to differentiate this species from other reptilian *Entamoebae*. In 1912, Alexeieff identified and figured *E. testudinis* from a Ceylon turtle, *Nicoria trijuga*. The approximate range of size (10·6–25·6 $\mu$ by 10·6–23·3 $\mu$, nucleus 4–8 $\mu$), calculated from his figures, is much smaller than the original dimensions furnished by Hartmann. Wenyon (1926) also found this species in 2 turtles, *Testudo argentina* and *T. calcarata*, but did not give any figures or measurements. We have examined stained preparations of *Entamoeba testudinis* from a turtle, *Chrysemys picta*, in the collection of Dr L. R. Cleveland, and believe that the species is closely related to or possibly synonymous with *Entamoeba serpentis* Cunha & Fonseca 1917. The host of *E. serpentis* was a snake, *Drymobius bifossatus*. Unfortunately, these authors failed to give measurements and no cysts were described, so that this species will be extremely difficult to identify. However, it is described as having a "smaller dimorphic form", but apparently either precystic stages or forms of an *Endolimax* were represented in their figures.

*Endamoeba* [=*Entamoeba*] *barreti* Taliaferron & Holmes 1924, from a snapping turtle, *Chelydra serpentina*, is also a distinct species. The trophic stages (from intestine 14·5–22·4 $\mu$, average 18·7 $\mu$; from culture 13·8–22·9 $\mu$, average 17·5 $\mu$; nucleus 5–6 $\mu$) were alone described. One of us (Q. M. G.) has obtained and cultivated 2 strains of an amoeba from *C. serpentina* and the organism has been identified as *Entamoeba barreti*. This species resembles *E. invadens* in cultural characteristics but produces distinctive 8-nucleate cysts in the mucin-saline-rice starch medium. Wenyon (1920) recorded a brief description of an *Entamoeba* sp. which he found in 2 lizards, *Lacerta agilis* and *Agama stellio*. He noted that it had a striking resemblance to *Entamoeba coli* in the trophozoite stage and that it produced an 8-nucleate cyst "which again is not distinguishable from that of the human *E. coli* either in size or characters". That *E. barreti* and *Entamoeba* sp. of Wenyon are distinct from *E. invadens* is certain, but further work (now in progress) on the life cycle of an *Entamoeba* producing 8-nucleate cysts will have to be completed before more detailed comparisons can be made.

During routine autopsies and blood examinations of animals dying at the Zoological Gardens in London, Plimmer (1916) reported amoebae in the blood of 3 snakes, *Tropidonotus viperinus*, from Europe, and 1 snake, *T. fasciatus*, from North America. No description of the amoebae was given, but since enteritis of reptiles is listed as a cause of death, it is possible that Plimmer saw several cases of amoebiasis in reptiles without recognising it as a distinct

disease. Since we found spontaneous amoebiasis in two species of *Varanus*, it is possible that Lavier (1928) found the causative agent of this disease of certain reptiles when he described *Entamoeba varani* (trophozoites 12–25 $\mu$, nucleus 5 $\mu$, no cysts) from *Varanus niloticus*. Lavier gave only a few figures of this organism, no average diameter of the trophozoites, and no range of size for the nuclei. The size is within the limits for *Entamoeba invadens*, but it also corresponds to some extent with that of *E. barreti*. Of the nuclear structure he states that it is identical with that of *E. dysenteriae*, but does not give a detailed description. Furthermore, he found no cysts, but states that the amoeba is easily recognisable in the two posterior parts of the intestine, though the appearance of the mucosa did not permit him to assign a pathogenic rôle to it. Consequently, in the absence of disease, this amoeba may be a distinct species; but Lavier's description is so brief that *E. varani* will be difficult to identify morphologically.

The first account of an amoeba in reptiles that produces 4-nucleate cysts was given for an *Entamoeba* sp. by Knowles & Das Gupta (1930). The description of the trophozoites (10–60 $\mu$, average 30·9 $\mu$; nuclei 5–6 $\mu$) and cysts (7·5–20·7 $\mu$, average 11·7 $\mu$) was based entirely on culture material obtained from a Gangetic turtle, *Trionyx gangeticus*. Although their descriptions are complete, these workers were fully aware of the uncertainties involved with the *Entamoebae* already described from reptiles, and did not name their own species. It differs from *E. invadens* in the following ways: (1) lighter staining peripheral chromatin in the nucleus (this agrees with Rodhain's but not our description); and (2) a smaller mean diameter of the cysts. These authors state that the amoeba they describe "has close resemblances to both *E. testudinis* and *E. barreti*", and that the evidence they have brought forward "rather tends to suggest that *E. testudinis* and *E. barreti* are synonymous, though the matter requires further investigation". However, when the distinctive nucleus of *E. testudinis* and the ripe cysts of *E. barreti* are considered, this *Entamoeba* sp. of Knowles and Das Gupta is more closely related to *E. invadens* than to either *E. testudinis* or *E. barreti*.

In our opinion, *E. terrapinae* Sanders & Cleveland 1930, is also a distinct species. The trophozoites, from the host (*Chrysemys elegans*) and cultures, are quite small and measure 10–15 $\mu$ with nuclei 2–4 $\mu$. This species forms quadrinucleate cysts (8–14 $\mu$, average 10 $\mu$) and the size and shape of the chromatoids are diagnostic. Moreover, this amoeba has the least complicated life cycle known for any species of the genus.

The most recently named species, *Entamoeba invadens* Rodhain 1934 (trophozoites from host 17–30·6 $\mu$, average 22 $\mu$, from culture, 21–25·5 $\mu$, nuclei 3·5–5 $\mu$, cysts 12·75–18 $\mu$) is described without giving the average diameter of the cysts and without adequate description and figures of cysts and trophozoites; hence, a complete morphological comparison with other species cannot be made. This author states that morphologically *E. invadens* is closely related to "*Entamoeba dysenteriae*", but then writes as follows: "Mais si l'on analyse

les détails de la structure du noyau, on remarque certaines particularités, notamment l'aspect du caryosome dans les préparations fixées au Bouin, et l'extrême délicatesse de la chromatine périphérique qui permettent de distinguer l'*Entamoeba dysenteriae* de l'*Entamoeba* décrite ici. Dans les cultures cette dernière se comporte très différemment de *Entamoeba histolytica*." The above points have not been found by us to differentiate the nucleus of this disease-producing amoeba of snakes from that of *E. histolytica*. We believe that the nature of the karyosome in preparations fixed with Bouin's fluid, and the extreme delicacy of the peripheral chromatin used by Rodhain to distinguish *E. invadens* from "*E. dysenteriae*" [*E. histolytica*] are mainly differences that could be brought about by excessive destaining or by the failure to wash out properly the picric acid of the fixative. To protozoologists familiar with the variations in morphology and size encountered in different strains of one species of *Entamoeba*, in a species of *Entamoeba* from a single host series, or from a pure line culture started from a single trophozoite or cyst, the nuclear characters of *E. invadens* given by Rodhain are extremely meagre for species identification. However, Rodhain also mentions that *E. invadens* will grow at laboratory temperature—in contrast with the rigid temperature requirements needed by *E. histolytica*; but we originally mentioned this fact (1934 *a*). Then, too, the pathogenicity of *E. invadens* as a character was not included in the description by Rodhain. We presented evidence (1934 *b*) in abstract for the pathogenicity of our strains of snake amoebae. The complete evidence will be published separately. In a paper adjoining Rodhain's description of *E. invadens*, Rodhain & van Hoof (1934) attempted to establish the pathogenicity of *E. invadens*, but their evidence is far from conclusive. They say that a *Varanus bengalensis* died of a disease similar to the one that proved fatal for a *Python sebae* and a *Rachidelus brazili*, yet no amoebae were found in the *Varanus*. Their experimental infections included 2 animals, a turtle (species?) and a *Tropidonotus viperinus*. The turtle was negative and the *T. viperinus* was positive when it died 15 days after inoculation. In a more recent paper, Rodhain & van Hoof (1935) have presented additional evidence to show the pathogenicity of *Entamoeba invadens*. This evidence—3 new cases of spontaneous amoebiasis, and experimental infections of 8 snakes of 2 species (*Tropidonotus viperinus* and *T. natrix*), 4 blindworms (*Anguis fragilis*) and 8 other lizards (*Lacerta muralis*), 3 turtles of 2 species (*Emys orbicularis* and *Testudo tabulata*), and 17 frogs (*Rana temporaria*)—indicates that their strains of *Entamoeba invadens* were pathogenic for snakes, lizards, and a turtle (*Testudo tabulata*). Nevertheless, it is evident that the species was founded on characters of no specific value—particularly for the genus *Entamoeba*.

Finally, the similarity of *E. invadens* and the possibility of synonymy with *E. ranarum* must be considered. Many workers have previously noted the similarities between *E. ranarum* and *E. histolytica*. Dobell (1918), in a series of experiments, failed to infect tadpoles with *E. histolytica* by feeding the cysts, and concluded that the two *Entamoebae* were "probably distinct

species". Sanders (1931) showed that the life cycle of *E. ranarum* was identical with that of *E. histolytica*, but assumed that the two species were distinct. We have examined preparations used by Sanders for this study, and consider the snake amoeba distinct from her strain of *E. ranarum* on the basis of morphology. Since the work of Dobell (1928) and Cleveland & Sanders (1930), the physiological evidence is sufficient to distinguish the frog *Entamoeba* from the dysentery amoeba of man. However, since we have discovered a pathogenic *Entamoeba* in certain reptiles, and thus far have failed to find a source of the infecting organism, the possibility has to be considered that the snakes got their infections from frogs which were fed to them at the Philadelphia Zoological Gardens. We examined approximately 3 dozen frogs from this source but failed to find a single *Entamoeba* infection. In work now in progress, one of us (Q. M. G.) has tried to produce amoebic disease in frogs with *E. invadens*. This work is not completed, but results indicate that an infection with *E. invadens* cannot be produced in frogs by the feeding of mature cysts. Apparently the trophozoites derived from a small proportion which excyst cannot survive the environment in the small intestine. Intraperitoneal injections have been negative, but intrarectal injections of cultures, both *via* the anal opening and through the wall of the rectum after obstruction of the anus, have served to establish the organism in the lumen of the rectum, but resulted in no disease or tissue invasion. Amoebae recovered by culture from these infections have retained their pathogenicity. Thus, results indicate that *E. invadens* may be distinct from *E. ranarum*, but cross-infection experiments with the frog *Entamoeba* will have to be performed before they can be considered conclusive. It is certain that the normal physiological environments of *E. invadens* and *E. ranarum* approach each other more closely than the normal environment of either approaches that of *E. histolytica*.

A careful comparison of the morphology and life cycle of *E. invadens*, as presented above by us, with the morphology and life cycle of *E. histolytica* reveals striking similarities. The structures of the trophozoites and cysts of the two amoebae are similar with only minor differences in *E. invadens*, such as greater density of cytoplasm in the trophozoites and a thicker wall on the cysts to distinguish it from *E. histolytica*. A major difference between the nucleo-cytoplasmic ratios appears to be more significant. The average ratio for *E. invadens* is 0·295 in contrast with the following values given by various workers for *E. histolytica*: Hegner & Chu (1930), man 0·22, monkey 0·22; Hegner, Johnson & Stabler (1932), monkey 0·267, man (case 3) 0·2775 and 0·2493, and man (case 1) 0·246. The ratio obtained for *E. histolytica* by us from measurements made on preparations from a hospital case was 0·249. The average ratio for all these strains of *E. histolytica* is 0·246, a lower ratio than we found for *E. invadens*.

The physiological differences between these two pathogenic *Entamoebae* are possibly of greater importance than the minor morphological differences, yet they may be primarily the result of physiological differences between the

two hosts. *E. invadens* is very constant in its structure owing possibly to its ability to live and grow over a relatively wide range of temperature. The dysentery amoeba of man quickly degenerates unless maintained in a suitable medium or host within the narrow temperature range of 35–38° C. Excystation requires the same conditions, but the cysts will survive in a liquid medium at a much lower temperature, 3–7° C., than that required for the growth of trophozoites. In culture, Dobell (1928) usually obtained excystation for *E. histolytica* in 3–5 hours; Cleveland & Sanders (1930) state that the majority of cysts excyst between 4 and 6 hours; Hegner, Johnson & Stabler (1932) observed excystation of *E. histolytica* 2–3 hours after cysts had been injected into the stomachs of 2 brown howler monkeys; and Tanabe (1934) obtained the excystation of cysts of *E. histolytica* 3–7 hours after feeding them to white rats. These results agree closely, but the time is less than that required for the same process when cysts of *E. invadens* are inoculated into snakes. In this species, the minimum time for excystation is approximately 5 hours after inoculation, but the process can occur up to 14 hours. The metacystic development of *E. histolytica* requires only about 7 hours under favourable conditions (Dobell, 1928; Tanabe, 1934), whereas the greater portion of this process in *E. invadens* takes place 12–24 hours after inoculation. Even though the requisite physiological environment for excystation and metacystic development in these two species of pathogenic amoebae is apparently quite different, the difference in time necessary for the similar processes is an actual fact and serves as a basis for comparison which may differentiate the two species.

The similarity between the morphology of these two species causing amoebiasis in their respective hosts tends to create the strong suspicion that the two species are identical. If one would assume that the differences in time needed for excystation and metacystic development are due to the different temperatures at which these species produce disease and are therefore insignificant, it may be considered justifiable to call the species described above a variety of *E. histolytica*. Further experimental evidence may eventually make this step necessary. In fact this step has already been taken for the *histolytica*-like amoebae of certain mammals, Chiang (1925) for an amoeba of the white rat (species?), Dobell for the amoebae of certain monkeys, and Nagahana (1934) for an amoeba of the wild rat (*Mus norvegicus*). On the other hand, since the physiological gap between the two host environments of these pathogenic *Entamoebae* is very great according to present opinions, since it is a matter of convenience to use specific names instead of varietal names, and since Rodhain was dealing with a pathogenic organism, we wish to apply the name *E. invadens* to the organism which we have described, rather than name the organism a variety of *E. histolytica* or erect a new species because of the inadequacies of Rodhain's description.

### DIAGNOSIS OF *ENTAMOEBA INVADENS*

Trophozoite: size 9·2–38·6$\mu$ × 9–30$\mu$, average diameter 15·9$\mu$; motility rapid in one direction in slug-like manner with explosive protrusion of clear ectoplasmic pseudopodia upon change of direction; feeds on leucocytes, liver cells, cell débris, bacteria, and starch grains; produces lesions in the alimentary tract (stomach, duodenum, ileum, and colon) and the liver; nucleus 3·5–7·3$\mu$, average diameter 4·78$\mu$; peripheral chromatin granules evenly distributed under thin nuclear membrane; central karyosome surrounded by small halo and "achromatic cloud"; nucleo-cytoplasmic ratio in different strains 0·26–0·34, average 0·295. Cyst: size 11–20·2$\mu$, average diameter 13·88$\mu$; 1–4 nuclei; glycogen vacuole; chromatoids acicular, rod-like, or cylindrical. Encystation and formation of quadrinucleate cysts in intestine or post-mortem in liver; time for excystation in snake's intestine 5–14 hours, majority 5–7 hours; time for metacystic development in snake's intestine 7–24 hours, majority 12–24 hours; temperature range for maximum growth in cultures 20–30° C.

Hosts (spontaneous amoebiasis in all cases): *Varanus salvator*, *V. varius*, *Tiliqua scincoides*, *Pseudoboa clelia*, *Lampropeltis getulus*, *Ancistrodon mokasen*, *Natrix rhombifer*, *N. sipedon*, *N. sipedon sipedon*, *N. cyclopion*.

Locality: Reptile collection, Zoological Gardens, Philadelphia, Pennsylvania.

Type: Gross mounts of diseased organs and stained slides of the amoeba and diseased tissue in permanent collection, Department of Comparative Pathology, Harvard Medical School; Laboratory of Comparative Zoology, Zoological Society of Philadelphia; and in private collections of authors.

### SUMMARY

The morphology and life cycle of an amoeba producing amoebiasis in reptiles is described. The organism is assigned to the species *Entamoeba invadens* Rodhain 1934, because of the pathogenicity of that species.

Material for the study was derived from 30 individuals of 7 species of reptiles, either dying of spontaneous amoebiasis or experimentally infected. (Material from 3 additional species of reptiles dying of amoebiasis was not used in this study.)

With the exception of a few minor morphological differences, *E. invadens* was found to be strikingly similar to *E. histolytica* in morphology of trophic and cystic stages, and in the processes of encystation, excystation, and metacystic development.

Metacystic development was studied in stained preparations made from the intestines of snakes 1–24 hours after the inoculation by mouth of mature cysts. Excystation usually takes place in the jejunum and ileum, a single quadrinucleate amoeba hatching from each cyst. Each quadrinucleate metacystic amoeba ultimately forms 8 amoebulae as a result of one division of each

cystic nucleus, cytoplasmic division, and parcelling out of the cystic (*N*) and daughter (*n*) nuclei. Nineteen of the 24 possible combinations of *N* and *n* nuclei were observed.

The culture phenomena of multiple fission without encystment is suggested, and cannibalism of cysts by the trophozoites is described.

The relation of *E. invadens* to the following species of *Entamoeba* from reptiles is discussed: *E. testudinis* Hartmann 1910; *E. serpentis* da Cunha & da Fonseca 1917; *E. barreti* Taliaferro & Holmes 1924; *Entamoeba* sp. Wenyon 1920; *E. varani* Lavier 1928; *Entamoeba* sp. Knowles & Das Gupta 1930; *E. terrapinae* Sanders & Cleveland 1930. The relationship and possible synonymy of *E. invadens* with *E. ranarum* and *E. histolytica* are also considered.

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## EXPLANATION OF PLATES VIII AND IX

All drawings are × 1570 and were made with the aid of a camera lucida from prepared slides fixed with Schaudinn's fluid plus 5 per cent. of glacial acetic acid (unless otherwise noted), and stained with Heidenhain's iron-alum haematoxylin.

### PLATE VIII

All figures are of *Entamoeba invadens* with the exception of Fig. 8 which is of *E. histolytica*.

Fig. 1. A trophozoite with typical, rounded, clear ectoplasmic pseudopodia from the intestine of *Natrix rhombifer*. (Fixed with half strength Schaudinn's fluid plus 2 per cent. of glacial acetic acid.)

Fig. 2. Trophozoite from a culture of strain *A*. The peripheral chromatin is packed tightly against the nuclear membrane.

Fig. 3. Trophozoite in liver smear from *Coluber constrictor*. Note the remains of degenerated liver cell nuclei to the left of the nucleus. The deeply stained cytoplasmic bodies are believed to be the remains of corpuscle nuclei.

Fig. 4. An elongate slug-like trophozoite in liver smear from *Natrix rhombifer*. Occasionally a tuft of débris may be seen on similar individuals in life. (Fixed with Bouin-Grassé solution.)

Fig. 5. Trophozoite from a 7-day culture. Note chromatoids.

Fig. 6. A giant trophozoite from a culture of strain *A*, showing chromatoids and nuclei (*Nn*).

Fig. 7. Cannibalism of cysts by a strain *A* trophozoite in culture. Cyst nuclei are degenerating.

Fig. 8. A typical trophozoite of *Entamoeba histolytica* from a fresh diarrhoeic stool. Note similarities between the nucleus of this organism and those of Figs. 1–4.

Fig. 9. A precystic amoeba from a liver smear of *Natrix rhombifer*. The large nucleus and the secretion of a cyst wall can be seen, but no glycogen vacuole and chromatoids.

Figs. 10–17. Cyst of *Entamoeba invadens* in the liver and culture from the same host, *Natrix rhombifer*. (Figs. 13, 15 and 16 were fixed with half strength Schaudinn's fluid plus 2 per cent. of glacial acetic acid.)

Fig. 10. Uninucleate cyst in the liver with glycogen vacuole and chromatoids.

Fig. 11. Uninucleate cyst from an 8-day culture with smaller chromatoids.

Fig. 12. Uninucleate cyst with the nucleus dividing.

Fig. 13. Binucleate cyst in the liver.

Fig. 14. Binucleate cyst from a 7-day culture.

Fig. 15. Binucleate cyst with both nuclei dividing.

Fig. 16. Cyst showing two mature cystic nuclei and the division of a nucleus to form the other two nuclei.

Fig. 17. Quadrinucleate cyst from culture, showing the glycogen vacuole.

Fig. 18. A slightly immature cyst from the ileum of a *Natrix cyclopion*, 14 hours after inoculation with cysts.

## PLATE IX

All figures are of excystation and metacystic development of *Entamoeba invadens*.

Figs. 19–23. Stages in the process of excystation of *E. invadens* in the jejunum and ileum of snakes approximately 5 hours after inoculation by mouth with mature cysts.

Fig. 19. The beginning of excystation by the protrusion of protoplasm through a small opening in the cyst wall.

Fig. 20. One nucleus passing through the opening in the cyst wall.

Fig. 21. A stage that corresponds to an observation made on a living cyst during the process of excystation and figured in Text-fig. 1. One of the cystic nuclei has emerged through each of two small openings in the cyst wall.

Fig. 22. Two cystic nuclei have emerged through one opening.

Fig. 23. Three cystic nuclei have emerged. A varying amount of cytoplasm occasionally remains in the cyst after the 4 *N* trophozoite frees itself from the empty cyst.

Figs. 24–39. Stages in the metacystic development of *E. invadens*. The time after inoculation of mature cysts is given for each stage.

Figs. 24–25. Two 4 *N* metacystic amoebae with the nuclei clumped in a typical manner. Bacteria have been ingested. 5 hours.

Fig. 26. *NNNN* amoeba, with one nucleus dividing to form *NNNnn*. 12 hours.

Fig. 27. *NNN* amoeba, with one nucleus dividing to form *NNnn*. 12 hours.

Fig. 28. *NNnn* amoeba. Note relative size of nuclei. Original *N* nuclei have grown in diameter. 12 hours.

Fig. 29. *Nnnn* amoeba. 12 hours.

Fig. 30. *Nnn* amoeba. 12 hours.

Fig. 31. *NN* amoeba. 12 hours.

Fig. 32. *Nnnnnnn* amoeba. 18 hours.

Fig. 33. Amoeba with 8 *n* nuclei. This stage and the previous one rare. 12 hours.

Fig. 34. *nn* amoeba. 12 hours.

Fig. 35. *Nn* amoeba, with *N* dividing to form *nnn*. 12 hours.

Fig. 36. A typical amoebula. 12 hours.

Figs. 37–39. Various sizes of uninucleate amoebae found 24 hours after inoculation. Fig. 39 is a typical and full-grown *E. invadens* trophozoite resulting from the excystation and metacystic development of mature cysts in a snake.

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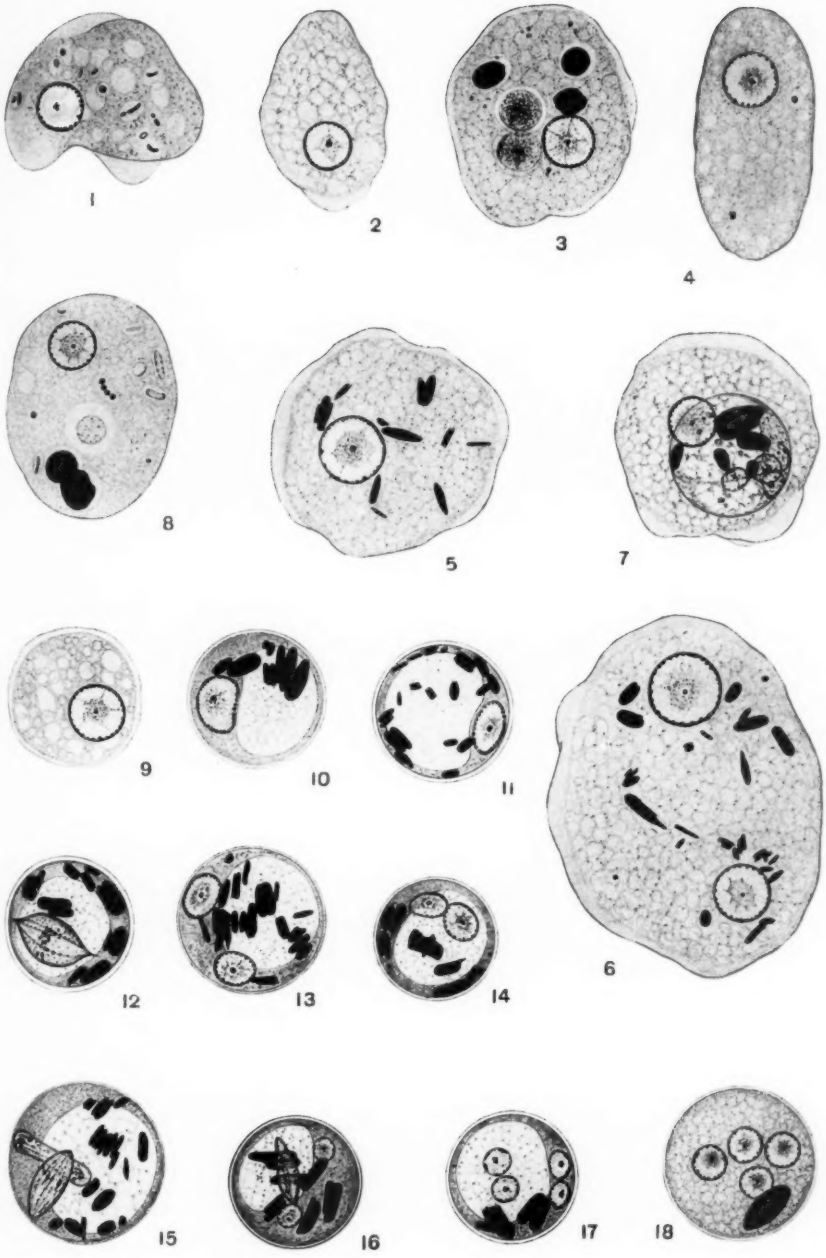


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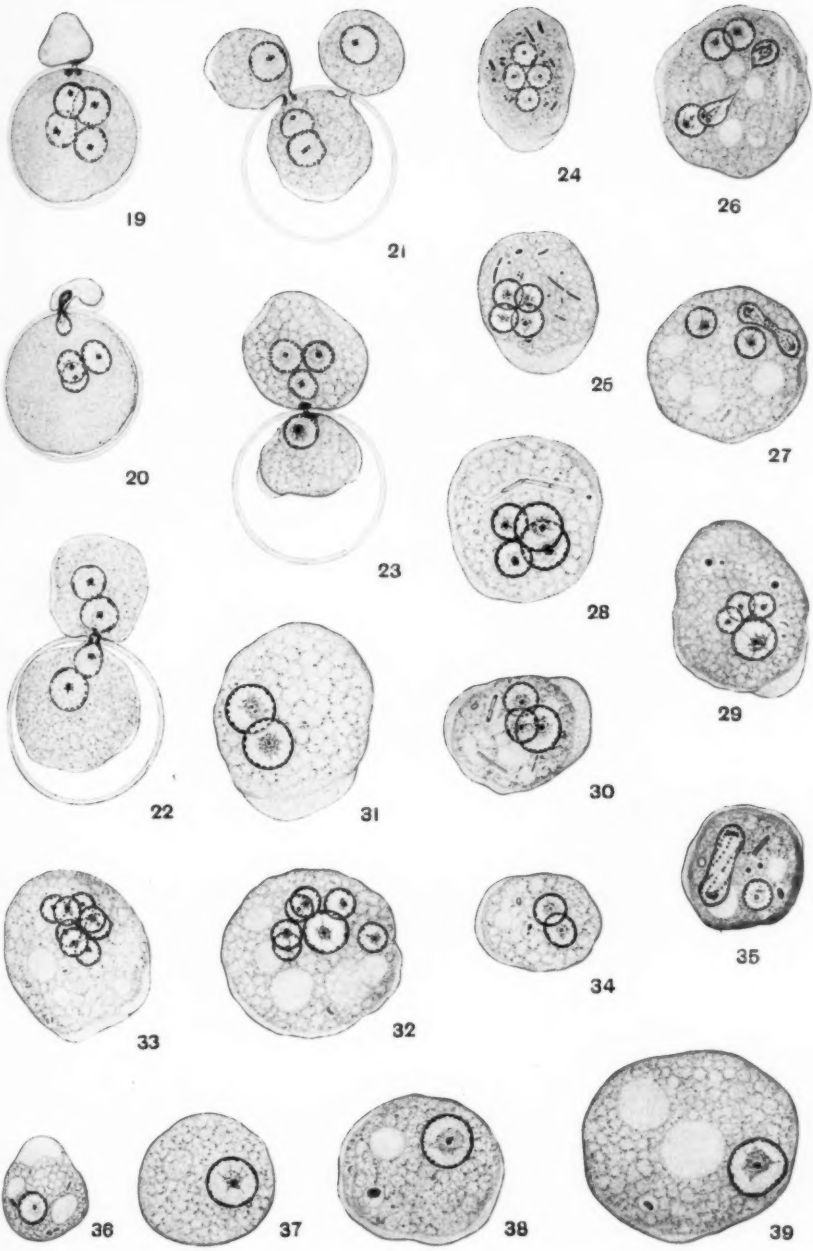


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# THE MORPHOLOGY AND DEVELOPMENT OF THE MONOGENETIC TREMATODE *SPHYRANURA OLIGORCHIS* (ALVEY 1933) AND THE DESCRIPTION OF *SPHYRANURA POLYORCHIS* N.SP.

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(With Plates X–XI, containing Figs. 1–16)

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## I. Introduction

Knowledge of the development of monogenetic trematodes is limited, since only four complete life histories have been described. Fuhrmann's (1928) statement, "Über die Embryonalentwickelung der Monogenea sind unsere Kenntnisse äusserst unvollständig", clearly points out the lack of information concerning the development in this group. No particular effort has been made to compare the successive developmental stages in members of the group for which the life cycle has been described. The study of these worms has been neglected for the more complicated life cycles of the Digenea, due perhaps to the somewhat erroneous impression that the Monogenea have a simple, direct development and that further investigation would contribute little to the understanding of the biology or taxonomic relations of the trematodes. The studies of Zeller (1872–6) on *Polystoma integerrimum* and their subsequent confirmation by Gallien (1932), of Kathariner (1904) on *Gyrodactylus elegans*, and the later work of Jahn & Kuhn (1932) on *Epibdella melleni*, have shown that the development of monogenetic species is not as simple as previously believed.

Very little is known about the phylogeny of the Monogenea. Fuhrmann (1928) stated that they are probably derived from a rhabdocoele ancestor. A phylogenetic arrangement of the rhabdocoele families was made by Meixner (1926) who traced their evolution, beginning with the Hyploblepharinidae through the Grafillidae and Anoplodiidae, to the Temnocephalidae which may be closely homologised with the Monogenea. Lönnberg (1897) did not concur in this view, and instead derived the trematodes from triclad turbellarians. Other workers believe that monogenetic and digenetic trematodes have a common ancestry. In view of the controversial status of trematode origin and of the relative position of the Monogenea and Digenea, it seemed that further studies on the development of the Monogenea and a comparison of corresponding stages in the group might reveal a common larval form which would give valuable information concerning their phyletic relations.

A survey of the literature shows studies on the life history of only four species: *Gyrodactylus elegans* (Kathariner, 1904), in the Monopisthodiscinea; *Epibdella melleni* (Jahn & Kuhn, 1932), in the Monopisthocotylinea; and two species, *Diplozoon paradoxum* (Zeller, 1872 *a*), and *Polystoma integerrimum* (Zeller, 1872), in the Polyopisthocotylinea. Other fragmentary observations have been reported, but they are too incomplete to be very significant. Of the four life histories known, one, *Epibdella melleni*, is of a marine form, and the others pertain to fresh-water species. The life histories of the two species of the Polyopisthocotylinea are so unusual that further work on a fresh-water form from this suborder is especially desirable.

A study of the life history of *Sphyranura* was undertaken, since this form is a common fresh-water polyopisthocotylean of uncertain position, ectoparasitic on the urodele *Necturus*, a host not previously considered in life-history work. It is believed that the results will prove helpful in an attempt to determine, not only its relation to the polystomes, but also the interrelations of the Monogenea as a group.

The systematic position of *Sphyranura* has long been a disputed question. Monticelli (1903) removed *Sphyranura* from the Polystomidae on the basis of the number of suckers. Odhner (1912), on the basis of internal anatomy, argued for its restoration to this group, and Fuhrmann (1928) reassigned it to the Polystomidae. A comparison of the development of *Sphyranura osleri* and *Polystoma integerrimum* should aid in determining their relationship.

I wish to thank Prof. H. W. Stunkard, under whose direction this work was undertaken, for his many helpful suggestions and for the loan of specimens of *Sphyranura* from his private collection.

## II. Historical survey

### A. *Morphology of* Sphyranura

An ectoparasite of the perennibranchiate amphibian, *Necturus maculosus*, was described by Wright (1879). His original description was made from preserved specimens together with certain sketches and observations on living

worms received from Prof. Osler. The form resembled the Polystomidae in (1) size and shape of egg, (2) structure of the suckers, (3) the shape and number of caudal hooks; and differed in (1) general form, (2) number of suckers, (3) structure of the genital apparatus. Wright proposed for it the generic name *Sphyranura* with the following characteristics:

"Body depressed, somewhat elongate, expanded posteriorly into a caudal lamina, considerably wider than the body, bearing two immersed acetabula, two large hooks behind these, and sixteen small hooks (seven along each side of the lamina and one in the centre of each acetabulum). Mouth ventral anterior, somewhat funnel-shaped, intestine with two branches anastomosing posteriorly. Excretory pore between the acetabula, two contractile bladders anteriorly, oviviparous. Parasitic on the gills of perennibranchiate Amphibia."

For the specific name he proposed *osleri* with the following diagnostic characteristics:

"Body 2·6–4 mm. in length by 0·7 mm. in breadth, narrowed at each end, especially where it joins the caudal lamina, which measure 1 mm. across, and about 0·45 mm. in length. Large hooks 0·2 mm. long. Oviduct occupying the interval between the intestinal caeca with numerous eggs; uterus with a single mature egg, oval, with brownish yellow shell, 0·364 mm. × 0·247 mm."

Wright believed *Sphyranura osleri* to represent a transitional form between *Gyrodactylus* and *Polystoma*, and pointed out that the larva of *Polystoma* resembled that of *Gyrodactylus* sufficiently to indicate a gyrodactylid ancestry. He suggested that the genera *Polystoma*, *Gyrodactylus*, and *Sphyranura* should be placed in the family Gyrodactylidae of van Beneden & Hesse (1863), since *Gyrodactylus* was apparently nearer the main stem.

Because of the lack of adequate fresh material for Wright's original description, Wright & Macallum (1887) redescribed *Sphyranura osleri* and made certain observations on its finer structure. The generic and specific characters were also changed as follows:

"*Sphyranura*. Body not depressed. Two contractile bladders anterior each with a dorsal excretory pore. No lateral vaginae."

"*Sphyranura osleri*. Lobules of testis, 12–16 in number, occupying the interval between the intestinal caeca, which anastomose posteriorly."

They revised the diagnostic characters of the subfamily Polystomidae, Taschenberg (1879), to include the genus *Sphyranura*.

Monticelli (1903) revised the classification of the Monogenea on the basis of adhesive apparatus and erected a new family, Dicotylidae, to contain *Sphyranura*. This classification was rejected by Odhner (1912) who used, as taxonomic criteria, differences in the female reproductive ducts. Among the Monogenea, Odhner separated the genera into two groups: Monopisthocotylea, characterised by a true vagina, no genito-intestinal canal and a single posterior organ of attachment; and Polyopisthocotylea, characterised by a *ductus vaginalis*, a genito-intestinal canal, no true vagina, and numerous posterior organs of attachment. He considered the seminal receptacles of *Sphyranura* homo-

logous to the paired vaginae of *Polystoma* and restored the genus *Sphyranura* to the Polystomidae in the Polyopisthocotylea. It was pointed out by Stunkard (1917) in his review of the literature on the classification of the Monogenea that Odhner, in maintaining the homology of the true vagina with the genito-intestinal canal, invalidated the basis of his (1912) classification into suborders. Stunkard agreed with Odhner that the seminal receptacles of *Sphyranura* are homologous with the paired vaginae of *Polystoma*, and that Monticelli's removal of *Sphyranura* from the Polystomidae on the basis of the difference in the number of suckers was not justified. Both Odhner and Stunkard noted that the name Dicotylidae was invalid, since it did not conform to the rules of zoological nomenclature. Poche (1925) adopted the name Sphyranuridae and included the family in the suborder Polyopisthocotylea.

### B. *Development of Monogenea*

As previously stated, the knowledge of the life history of the Monogenea is extremely limited. In most of the Monogenea, development is probably direct, since this is the case in those life histories which have been described. The larva at the time of hatching usually resembles the adult and grows without metamorphosis into the final stage. On hatching, it usually possesses cilia which are arranged either in bands or tufts at definite positions on the body. After a free-swimming period the larva becomes attached to the host, loses its cilia, increases in size, and gradually assumes the adult form.

The first observations on the development of the Monogenea were made by Zeller (1872) on *Polystoma integerrimum*. He found that the eggs are deposited infrequently in the water where they develop into a multinucleate mass which undergoes peripheral differentiation to form the larva. Five incomplete bands of cilia are formed, three near the anterior end and two near the posterior end. At the time of hatching the larva possesses four eye spots, an intestine, a well-developed excretory system, and at the extreme posterior end a caudal disc with sixteen larval hooks and no suckers. After a short free-swimming period it adheres to the gills of a young frog tadpole, the cilia then disappear, and the suckers develop in pairs around certain of the larval hooklets.

The larva makes its way down the alimentary tract where, on the formation of the cloacal bladder, it enters and remains for three years before becoming sexually mature. When a larva attaches to the external gills of a very young tadpole it does not migrate to the rectum but remains on the gills, matures, produces eggs, and finally dies upon metamorphosis of the host. Occasionally the external gill forms will develop in an internal gill. The structural differences between the two forms of *P. integerrimum* were studied by Zeller (1876). Willemoes-Suhm (1872) and Zeller (1872) pointed out the similarity of the early stages to those of *Gyrodactylus*.

Because of the unusual findings of Zeller, Stunkard (1917) suggested that he

might have confused two species. Gallien (1932 *a* and *b*, 1933), on reinvestigation of the problem, confirmed Zeller's observations. Gallien has discovered the existence of an alternation of generations, instead of merely a chance development based upon the age of the host at the time of the initial parasitism. He reports that worms of the bladder type give rise in the spring to eggs which hatch and develop into the gill type that matures in a few weeks. This form in turn produces eggs which hatch into larvae that adhere to older tadpoles and gradually develop into the bladder type after three years.

The life cycle of *Diplozoon paradoxum* was reported by Zeller (1872). The larva at the time of hatching is uniformly ciliated and possesses a pair of posterior suckers, two eye spots, a well-developed pharynx, and a blind sac-like gut. After a short swimming period it becomes attached to the host, sheds the cilia, elongates, enlarges at the posterior end, loses the eye spots, and develops a branched gut. In the mid-region of the body a ventral sucker and a dorsal cone are formed. This is the diporpa stage of Dujardin (1845). Two individuals come together and the mid-ventral sucker of one becomes fastened to the dorsal cone of the other, after which the worms bend around until the free ventral sucker adheres to the other dorsal cone. They fuse in this X-shape to form the adult Diplozoon stage of von Siebold (1851). Zeller (1888) has pointed out that the worms are permanently *in copula*.

A third type of life history is shown by the viviparous development of *Gyrodactylus elegans* reported by Kathariner (1904). The egg develops in the parent and gives rise to the daughter and granddaughter larvae. After development reaches the point at which the sixteen larval hooks and two spines are formed, the embryo passes out of a "birth opening" and becomes attached to the host. The daughter *Gyrodactylus* may have within its uterus two or three embryos which emerge before it reaches maturity. Each succeeding generation is smaller than the previous one. Kathariner concluded that this development was true polyembryony.

A more simple and direct type of development is found in the life history of *Epibdella melleni*, described by Jahn & Kuhn (1932). The swimming larva has a well-developed oral cup, simple digestive and excretory systems, two pairs of eye spots, three ciliated areas at the anterior, middle, and posterior regions, and a folded non-functional sucker at the posterior end. It has three pairs of spines and fourteen larval hooks which are located at the margin of the sucker and ventral to the spines. After attachment by the anterior sucker the cilia are lost, the posterior sucker unfolds, and the worm gradually assumes the adult shape.

The swimming larva of *Dactylogyrus crassus* and the early stages of *D. anchoratus* have been described by Kulwieć (1927). In *D. crassus* there is a free-swimming larva which has the cilia located in three bands on the anterior, middle, and posterior parts of the body. It has two pairs of eye spots, a muscular pharynx, and seven pairs of larval hooks which are not located in a definite adhesive disc but are fastened to the posterior part of the body.

Since she was unable to obtain the early larval stages, Kulwieć had to limit her description of *D. anchoratus* to its development on the gills. At this stage the adhesive disc with its fourteen hooks was present. From observations on these two forms Kulwieć traced what she believed to be the typical life history of the members of the genus. According to her the eggs are laid, the larvae develop and break forth from the egg as ciliated forms with two pairs of eye spots and a well-developed pharynx. The fourteen larval hooks are present but may or may not be supported on an adhesive disc. The larva adheres to the gills of fish, the cilia are lost, the adhesive disc develops with large hooks arranged in the centre. This is followed by a general increase in size, and in development of the individual to maturity.

Further observations on the Monopisthodiscinea have been made by Siwak (1931) who studied *Ancyrocephalus vistulensis*. The larva has three ciliated bands, a pharynx, and twelve larval hooks which are attached to the posterior body wall. After attachment to the host, following a swimming period, the larval hooks become rearranged in the developing adhesive disc; this is followed by the development of the large hooks in the centre of the disc. Siwak does not figure or discuss the later stages of development.

Our present knowledge of the genus *Sphyranura* is limited to the papers of Wright (1879), and Wright & Macallum (1887). Although a very common form, the genus apparently has not been studied since the publication of the two original papers.

## III. Material and methods

Specimens of *Necturus maculosus*, parasitised by a species of *Sphyranura*, were collected near Meadville, Pennsylvania. They were kept in small tanks to which water could be added and drawn off at the top. This made it possible to maintain a fresh supply of water without disturbing the eggs of *Sphyranura* which, on being laid, sink to the bottom. At regular intervals the tanks were cleaned out so that freshly laid eggs might be obtained.

Eggs were collected and placed in small watch-glasses convenient for stacking and observation under the binocular microscope. The water in the watch-glasses was changed frequently and the larvae were removed immediately upon hatching. Efforts to isolate and maintain worms which were depositing eggs were unsuccessful. The adult worms will live for only a short period after removal from the host and cease to deposit eggs.

Attempts were made to remove the *Sphyranura* by picking them off with forceps. This proved to be very difficult, as the majority of the worms are attached among the gills. Parasite-free animals were finally obtained by immersing the necturi in a fairly strong chloretone solution. After a variable period dependent on the concentration of the anaesthetic, the worms were loosened from the animal. This method also served the dual purpose of anaesthetising the necturi so that they could be examined closely. They were then returned to a parasite-free tank and kept under close observation. Fre-

quently this process had to be repeated before an animal was freed of parasites. The newly hatched larvae were introduced into the tanks of parasite-free necturi and were recovered at frequent intervals by use of chloretone solution. The young worms were so small that it was necessary frequently to kill the necturus in order to use a binocular microscope in looking for them among the parts of the gills.

Observations were made on both living and fixed material. Whole mounts, stained with paracarmine, borax carmine, and Delafield's haemotoxylin, were made of the various stages. The internal structures were studied from frontal, sagittal, and transverse sections cut at a thickness of 6 or 7 $\mu$ and stained with iron haematoxylin or Delafield's haematoxylin, and counter-stained with eosin. Slides of seven whole mounts were borrowed from the private collection of Prof. H. W. Stunkard. The specimens were stained in paracarmine, borax carmine, and alum cochineal. Three of these slides were in a poor state of preservation, but important points of variance with the description of Wright & Macallum (1887) could be checked. Two of the specimens were removed from the slides, destained, sectioned, and restained with haematoxylin-eosin. The sections, although poor, furnished additional information.

## IV. OBSERVATIONS

The specimens reported in this paper do not agree with the descriptions of *Sphyranura osleri* made by Wright (1879) and Wright & Macallum (1887). Accordingly they are regarded as two distinct species. *S. oligorchis* was briefly described and named in a preliminary paper (Alvey, 1933*b*). For the other species the name *S. polyorchis* n.sp. is proposed.

### A. *Morphology of* Sphyranura oligorchis

The worm is somewhat elongate, narrowed at the cephalic end, broadened in the region of the anterior testes, and narrowed again at the junction of the main body with the caudal lamina. The type specimen (Fig. 1) is 2·5 mm. long, and 0·3 mm. across the anterior and posterior ends of the body. The caudal lamina is 0·77 mm. wide.

*Organs of attachment.* The chief organs of attachment (Fig. 1) are the two large hooks and the two suckers of the caudal lamina. The powerfully developed suckers are centrally located in each half of the caudal lamina and are about 0·2 mm. apart. The protrusion and retraction of the suckers were observed in living specimens. In whole mounts, viewed from the ventral aspect, the suckers appear cup-shaped, sunk deeply into the parenchyma, and composed of two zones, basal and distal, marked off by a thickened invagination of the cuticula. A small hook is present in the centre of the basal zone. Sections through the suckers (Fig. 14) show that they are bounded by a cuticular membrane which is thick on the concave and thin on the convex face. The wall of the sucker is made up of fibres which have a supportive rather than a muscular function, and run between the cuticular membranes.

At one point the inner cuticular lining folds against the exterior cuticular lining and fuses, thus interrupting the continuity of the fibrous wall and dividing the sucker into basal and distal zones. This unfolding forms a joint-like arrangement which allows independent movement of the two parts of the sucker. The basal zone also has a small secondary cuticular thickening (Fig. 14) which does not completely interrupt the fibrous continuity, and allows a bending of the basal area of the cup. The action is more limited than that permitted by the first cuticular ring.

In the centre of the basal portion of the sucker there is a structure, described by Johnston (1912) as a connective tissue plug, on which the powerful retractor muscles are inserted. It bears the larval hooklet and the cuticular ring which surrounds it. Foreign cells were observed attached to these hooks, although it is questionable whether they have any functional importance in the adult. Muscles are attached to the distal and median parts of the suckers (Fig. 16), and a small band of muscles is inserted on the inner part of the basal zone of the sucker.

The large hooks are about 0·26 mm. long in the adult specimens and are located posterior to the suckers with the curved end lateral in position. The hook (Fig. 13) is composed of a shaft which is flattened and striated at the proximal end. It extends laterally to be joined by a broad, flattened projection, and following the junction, becomes circular, reduced in diameter, curves sharply upon itself, and ends in a point. Two striations occur where the hook decreases in diameter and becomes round. At the point of the curvature, the hook passes through the investing membrane to the exterior. Muscles are attached to flattened projections of the hooks.

In addition to the suckers and large hooks, there are sixteen small hooks arranged with seven on each side of the caudal lamina and one in each sucker. They are somewhat similar in shape (Fig. 15) to the large ones, and are on an average about 0·025 mm. long. In the small hooks the short, flattened projection is nearer the curvature which does not bend as sharply as in the large hooks. At the point of their perforation of the investing membrane they are surrounded by a thick chitinous ring which is thinner at one point to permit change of shape. Slender muscles are attached to the broadened proximal ends of the shaft and its projection.

*Cuticula.* The body is covered by a thick cuticula. It is a clear, homogeneous, non-nucleated layer which is separated from the circular muscles by a narrow, fluid-filled space. A subcuticular layer is not present. Because of the absence of the circular muscles in the region of the caudal lamina, the cuticula rests directly upon the parenchyma. It is thicker on the dorsal side of the worm than on the ventral side and is considerably thinner on the caudal lamina. Examination of fixed and living preparations showed that the cuticula is thinned and raised at regular intervals into sharp-pointed, cone-shaped evaginations. These projections are especially numerous in the region of the oral sucker. The narrow fluid-filled spaces between the cuticula and the circular

muscles are enlarged into the cavity of the cones. Upon contraction of the worm the cuticula is thrown into many minute folds.

*The musculature.* The musculature consists of a dermo-muscular sac, the muscles of the adhesive organs, and scattered dorso-ventral fibres. The muscles of the body wall consist of an external circular layer and an inner longitudinal layer. The intermediate layer of diagonal fibres, as reported for the polystomes, is lacking. Occasionally dorso-ventral parenchymal fibres can be observed. The longitudinal muscles form a much heavier layer on the ventral side. Certain of these ventral longitudinal muscles converge as they proceed toward the posterior end of the worm and unite into two distinct round bundles as they enter the caudal lamina (Fig. 16).

These muscles proceed posteriorly and attach to the broad, flattened projections of the shafts of their respective hooks. Certain of these ventral fibres diverge on entering the caudal lamina and attach to the anterior portion of suckers. The other muscles of the caudal lamina consist of a transverse band (Fig. 16) which runs across from the projection of one hook to the other, and a pair of muscles which originate in the median portion of the caudal lamina and are inserted on the hooks at their striated proximal ends. The two sets of muscles which are fastened to the projection of the shaft of the hook are used to set the hook, while the muscles which are attached to the ends of the shafts withdraw them. Across the anterior end of the caudal lamina there are a number of fibres which run from one side to the other and which are concerned in its movement.

*The parenchyma.* The parenchyma is definitely cellular in nature and is not uniform in character, but varies in different regions of the worm. Two types of cells are prevalent. Type 1 is a small cell, diameter 10–12$\mu$, with a heavily staining nucleus which occupies the greater portion of the cell. It usually has one and infrequently two small nucleoli and coarsely granular chromatin which is strongly basophilic. The cytoplasm takes acidic and basic stains lightly. Type 2 is a large cell, diameter 20–22$\mu$, with a large nucleus and a large amount of cytoplasm. The nucleus is vesicular, lightly basophilic, and has a single large nucleolus. The cytoplasm is acidophilic. There is a third type of cell occasionally present in the parenchyma. This cell has a very large nucleus which occupies nearly the entire cell. It has finely divided chromatin which stains lightly with basophilic dyes. In the posterior region of the worm the cells are more numerous, and in the caudal lamina between the suckers there is a densely packed area of cells (Fig. 14) which resemble type 3. These cells seem to constitute a definite structure but do not have a limiting membrane. Their function could not be determined. Scattered parenchymal fibres were present throughout the worm and were most prominent in the anterior region.

*Digestive system.* The digestive system consists of an oral sucker, prepharynx, pharynx and bifurcated intestine which anastomoses posteriorly. The oral sucker is terminal in position and very mobile. It is weakly developed

(Fig. 1), does not have an external limiting membrane, and in the posterior region is not sharply delimited from the parenchyma. Muscle fibres run from the lining of the oral sucker laterally to end in the external body wall and posteriorly to end in the parenchyma. These fibres are frequently joined together into bundles, especially the fibres running longitudinally. As a result, the sucker has the appearance of a network of muscle bundles interwoven with finer muscle fibres. A definite fibre pattern as found in suckers of the caudal lamina is absent.

In certain cross-sections a number of fibres were observed to pass from the body wall and insert on the wall of the prepharynx, thus giving the appearance of a limiting membrane. The oral sucker and pharynx are lined with cuticula which is continuous with that of the surface of the body. The fibres found in the oral sucker are contractile, and interspersed among them are a number of large cells with deeply staining nuclei which are similar to those reported by Stunkard (1917) as having a secretory function in the polystomes.

The pharynx (Fig. 1) is bounded by a definite limiting membrane and has an oval shape which varies with the stage of contraction. It is diagonally placed in the body and the lumen runs from the ventral region to a more dorsal position where it opens into the intestine. The pharynx is composed of radial fibres which extend from the external limiting membrane to the cuticula of the lumen. There are two layers of strongly developed annular fibres, one of which is located just inside the cuticula, and the other just inside the external limiting membrane. The strong contraction and elongation of the pharynx when the worm is stationary is probably accomplished, partially by the outer layer, while the inner one serves as a sphincter for the digestive tract. In the posterior region of the pharynx there are a number of large cells interspersed among the fibres. These cells have single nucleoli and granular chromatin which is strongly basophilic. No trace of any ducts leading from these cells to the lumen of the gut could be found. Stunkard (1917) described this same condition for the polystomes.

The intestine is composed of two diverticula (Fig. 1) which run from the pharynx laterad and then turn posteriad and continue to a point above the caudal lamina, where they turn mediad and anastomose. The diverticula are lobed but not branched. The endothelium consists of large columnar cells with granular cytoplasm and nuclei which lie at the basal end of the cell. The nuclei are oval in shape, reticular, have nucleoli, and are strongly basophilic. The distal ends of the cells are irregular and do not form a continuous smooth surface.

*Male reproductive system.* The testes, usually six in number (Fig. 1), are located one behind the other midway between the diverticula of the intestine in approximately the middle third of the body. Of 184 worms examined in whole mounts, 122 had six testes, 32 had five testes, 29 had seven testes and one had nine testes. It is quite likely that in some of the 29 worms in which

seven testes were counted, the anterior testis may have been a seminal vesicle, as it is almost impossible to distinguish it from testes in whole mounts. In most of the specimens which have five testes, a definite space occurred at some point in the series. The testes are oval, diameter 0·08–0·1 mm., when viewed in whole mounts from the ventral side, and are elongated dorso-ventrally when observed in cross-sections.

The vas deferens (Fig. 1) runs in the median line dorsal to the testes and communicates with each by means of a short tube. It is narrow at the posterior end and enlarged toward the anterior end. The posterior part, as well as the short connecting tubes, has a fibro-membranous wall. The vas deferens enlarges in the region just anterior to the cephalic testis to form a seminal vesicle which resembles a seventh testis when it is filled with genital products. The vas deferens continues anteriorly from the seminal vesicle, passes dorsal to the uterus and turns sharply ventrad, where it constricts to open again into a spherical cirrus sac, which terminates in a circlet of eight spines. The cirrus sac is composed of an outer layer of circular muscles enclosing longitudinal parenchyma cells upon which the spines are attached. These longitudinal cells protrude the cirrus with eversion of the spines. In the retracted condition the cirrus is surrounded by a deep depression. The cirrus sac opens into a common genital atrium. The structure of the genital atrium is such that the cirrus on eversion into the genital sinus would tend to close the opening of the uterus and prevent self-fertilisation.

*Female reproductive system.* The ovary is ovoid, about 0·08 × 0·1 mm. It is located on the left side of the worm and from its posterior end the oviduct (Fig. 1) passes posteriad and ventrad, after which it turns toward the median line and enters the ootype. The duct is continued again from the anterior end of the ootype as a coiled uterine canal which after a short distance becomes reduced and then enlarges to form the uterus, opening into the common genital atrium. From the ootype a duct passes posteriad where it divides, each half running anteriad and laterad to end blindly in a small solid enlargement at a level slightly below the bifurcation of the intestine. These enlargements are presumably homologous to the seminal receptacles of *Sphyranura osleri* as described by Wright & Macallum (1887). The genito-intestinal canal (Fig. 1) passes laterally from the ootype on the same side as the ovary, to open into the intestine. The genito-intestinal canal, in worms observed under a slight pressure, may be filled with yolk material which is being eliminated into the gut.

The oviduct, ootype, and uterine ducts have a fibro-membranous covering around the epithelial lining. The uterine duct at its constriction, before it widens to form the uterus, has a thickened muscular layer. The uterus is a wide elongated sac which extends from the level of the ovary to the common genital atrium. It has an inner epithelial lining and an outer fibro-membranous layer, and in the anterior region there is an additional intermediate layer of heavy circular muscles which, by a peristaltic movement, aid in the elimina-

tion of the egg. The anterior portion of the uterine duct and the entire uterus are surrounded by a mass of elongated cells which have slender processes that pierce the duct and the dorsal and ventral walls of the uterus. These cells are glandular in nature and are filled with secretory droplets.

The vitellaria (Fig. 1) consist of follicles which are located in the middle half of the body, extending from the ovary to the region of the anastomosis of the intestinal branches, encircling each intestinal branch. The follicles are large and consist of several cells with heavily staining nuclei. A vitelline cell can easily be distinguished by the presence of a characteristic nucleolus which is large, oval, and stains a reddish blue colour. The cytoplasm of inactive cells is relatively compact, while in actively secreting cells it becomes distended and vacuolated. Numerous yellow yolk droplets appear in the vacuoles. The method of yolk collection was not determined.

The genital pore (Fig. 1) is located on the ventral surface in the median line just posterior to the bifurcation of the intestine. It opens from a common genital sinus into which the uterus and cirrus empty. The opening of the uterus into the genital sinus is posterior and ventral, while the cirrus opens into the dorsal part and can be extruded from the pore. A number of worms were placed in a watch-glass and observed for a considerable period of time, but they failed to show any tendency to copulate. The eggs (Figs. 3, 5), 0·28–0·41 × 0·22–0·26 mm., are oval, operculate, and have a brownish yellow shell.

*Nervous system.* The most conspicuous portion of the nervous system consists of two large, lateral, ventral cords located on either side of the worm, paralleling the course of the intestine. In the anterior region they are connected by a suprapharyngeal dorsal commissure, and in the posterior region by a very large dorsal commissure in the caudal lamina. The nerve cords and caudal commissure are more strongly developed in the posterior than in the anterior end. This is probably correlated with the development of powerful adhesive organs at the posterior end.

*Excretory system.* There are two large contractile bladders (Fig. 1) located in the anterior end of the worm lateral to the intestinal branches and just posterior to the level of the genital pore. Small dorsal pores open to the exterior from the anterior region of the bladders. The walls of the excretory vesicles are composed of fibro-muscular tissue. Contraction of the bladders usually occurs alternately.

A duct opens posteriorly from each bladder and runs to the region of the caudal lamina, where it gives off several small branches and then doubles back on itself to run in close proximity to its former path. It passes around the bladders and breaks up into small branches in the anterior end. Very little of the flame cell pattern could be determined, although numbers of worms were studied for long periods under oil immersion. The flame cells observed were very long and difficult to distinguish from the branches of the excretory system.

*Vascular system.* A vascular system was observed which consists of a dendritic lymph sinus located in the anterior end around the oral sucker, and two lateral branches which run posteriad, one on each side of the worm, to terminate a short distance from the caudal lamina.

*Ecological observations.* The worms appear on the host in rather definite cycles of about seven to ten days' duration. There may be a heavy infestation one week and a week later only a very few worms. A period of heavy infestation occurs every six to ten weeks. When a necturus is heavily parasitised it secretes a large amount of slime and a slime coat is shed over the entire surface of the body and many of the worms are lost. When the necturi were fed with fresh liver the worms detached themselves and swam or crept on the bottom of the tank to the liver. If the liver is stale they are not attracted. In both the adult and larval stages, worms swim by means of the caudal lamina. Despite the fact that they would adhere to fresh liver, no success was obtained in attempting to place them on frog tadpoles. The worms, when placed on the tadpole, remained for only a few moments and then dropped off. Tadpoles of *Rana clamitans* kept in the tank with parasitised necturi did not become infected.

### B. *Development of* Sphyranura oligorchis

*Spermatogenesis.* The testis consists of a medullary and a parietal part. The parietal zone is composed of a single layer of cells which are of uniform size and structure with very large, deeply staining nuclei and a small amount of cytoplasm. Occasionally one of these cells can be seen in the process of division, although in the adult the number of dividing parietal cells is never very large.

In the central zone all of the various stages of development may be found. The parietal cell divides; one of the resulting cells becomes free, and by repeated division the spermatozoa are formed. The early stages of spermatogenesis could not be followed. At the spermatid stage the cells, some forty-five of which have been counted, are arranged in a sphere. They are wedge-shaped and radiate from the centre to the periphery near which the nuclei are located. As development continues the cavity enlarges, the cell boundaries disappear, and the nuclei elongate. The elongation of the nuclei continues until they extend from the periphery to the centre of the sphere and the cytoplasm completely disappears. The spermatids may be dispersed through the testis or remain in position and differentiate into spermatozoa. The end of the spermatid nearest the centre elongates to a thread-like form and the other end differentiates into a pear-shaped head. The spermatozoa now leave the testis and go to the vas deferens where they are collected in the seminal vesicle. Spermatids were frequently observed in the seminal vesicle, and consequently it may be concluded that occasionally final differentiation may take place in the seminal vesicle. The anterior portion of the vas deferens is always filled with a mass of spermatozoa in readiness for copulation. Although

copulation was not observed, it is difficult, in view of the structure of the genital atrium, to understand how self-fertilisation could take place.

*Oogenesis.* The ovary is surrounded by an outer limiting membrane of fibrous tissue. An occasional flat, elongated nucleus can be seen in the fibrous tissue of the limiting membrane. The ovary contains two types of cells. On the inner surface of the membrane at the antero-dorsal end of the ovary there is a crescent-shaped layer of parietal cells which have large nuclei with coarsely granular chromatin. Nucleoli could not be observed. These cells resemble the parietal cells of the testis. Division of the parietal cells takes place to form the oogonia which occupy the remaining portion of the ovary. After division of a parietal cell one of the daughter cells moves posteriad in the ovary and undergoes changes. As the developing ova reach the centre of the ovary they become compressed with their long axes at right angles to the long axis of the ovary. As they pass posteriad, the cells increase in size, with a corresponding increase in the size of the nucleus. The granular chromatin takes a homogeneous stain. The nucleolus appears with a single vacuole. At the extreme posterior end of the ovary there is always one spherical ovum which is ready for fertilisation. Penetration of the spermatozoa takes place at this point, and the polar bodies are probably formed later. The chromosome behaviour could not be followed in oogenesis.

*Fertilisation and formation of egg.* Insemination takes place in the posterior end of the ovary itself. Spermatozoa have frequently been observed between the ripe ovum and the anterior portion of the ovary. In a number of sectioned worms the ovarian end of the oviduct and the posterior end of the ovary were filled with spermatozoa. There is no seminal receptacle and no spermatozoa were ever observed in the small vestigial lateral ducts which are presumably homologous with seminal receptacles in *S. osleri*.

After fertilisation, the ovum passes down the oviduct, through the ootype, and into the uterus where the yolk material and shell are added. Although this process has not been followed in its entirety, it seems unlikely that the yolk and shell could be added in the ootype, as it is just large enough to permit the passage of the fertilised ovum. Sections through the uterus have been obtained which show only the ovum and yolk globules before the deposition of a shell. The fully formed egg, after the shell has been laid down, remains for some time in the uterus, and then passes through the genital atrium and pore, and settles to the bottom. The eggs (Fig. 3) when liberated are 0·28–0·41 mm. long. About 5 per cent. of them have a small projection at the narrower end, which is probably homologous with the polar threads of eggs in certain other monogenetic trematodes.

*Development of the embryo.* Whether cleavage occurs before the egg enters the uterus could not be determined. In only one case, as previously mentioned, was the ovum observed after it had left the ovary and before the shell was laid down. In this instance cleavage had not taken place. Sections of an egg, whose formation in the uterus of a worm had been followed under a

microscope for a period of two hours and ten minutes, showed a number of cells embedded in the yolk globules (Fig. 4). Development of the egg has been observed from the time the yolk mass appeared in a plastic state, capable of changing shape as contraction of the uterus took place, until it assumed the final shape of the egg. At this stage the cells of the developing larva are small, with a small amount of cytoplasm, and have nuclei containing coarsely granular chromatin and large homogeneous nucleoli. Little variation in structure was noted in a number of sections through various uterine eggs. The average egg production of isolated worms is about one egg every three hours, although eggs have been observed to remain in the uterus for as long as four hours. The significance of these observations is questionable, since they were made on worms isolated in watch-glasses and therefore under abnormal conditions.

The eggs when laid settle to the bottom and undergo development. The greater portion of the early embryonic and larval stages were studied in eggs collected from the debris that is always present in the bottom of the tanks. Eggs were isolated by means of a small pipette, placed in watch-glasses, and observed from day to day under a compound microscope. All attempts to fix and section these eggs after they had left the uterus were unsuccessful, due to the extremely brittle nature of the shell. The shell can be removed by immersing the eggs in $Ba(OH)_2$ for sixty seconds, but the resulting injury to them is so great that when sectioned they are of little value. In the thinner shelled eggs development could be followed under the microscope with little difficulty.

Cleavage continues at room temperature for fifteen to eighteen days. At this time a mass of cells has been formed and the number of yolk globules has been reduced. At about this time organisation of the cell mass can be observed, and a definite shape is assumed by the end of the twenty-second day. The embryo is U-shaped with the membrane complete at the anterior end, while at the other end it grades off into a formless mass of cells and yolk. By the end of the twenty-fourth day the embryo seems to be entirely surrounded by a delicate limiting membrane. Attempts to remove the shell with dissecting needles were unsuccessful, as the slightest pressure causes a rupture of the membrane.

The posterior end of the embryo now develops at a rapid rate, and the typical flattened caudal disc appears by the twenty-sixth to twenty-eighth day. At this time the embryo is folded with the anterior and posterior regions toward the operculate end of the egg which is characteristic of the later stages. Up to this point no activity has been manifested. At this stage of development, it was possible to remove the shell with fine needles, liberating the embryo into the water. On being freed they displayed very weak movements and many of them failed to straighten themselves. Straightened specimens were about 0·23 mm. long. Embryos at this stage were fixed in a straightened condition and some of them were sectioned.

In whole mounts they appear elongated and of uniform width except in the region of the caudal lamina which is wider than the rest of the body. The body of the embryo has no visible definite structures except in the caudal lamina which has sixteen small and two large hooks. The small hooks (Fig. 1) are arranged seven on each side and two in the centre of the caudal lamina. No cuticular rings of external openings could be discovered either in sections or in whole mounts. The large hooks, 0·09 mm. long, are ventral in position with their shafts pointing mediad and their curvature laterad. The hooked ends were open to the exterior, but it was impossible to tell whether this was a natural condition or due to the rupture of the tissues. The shafts of these large hooks (Fig. 11) seem to end in a bifurcation. No caudal suckers were observed.

Examination of sections (Fig. 6) showed a cuticula which encloses a mass of cells loosely bound together. These cells are of two distinct types. One type has a large vesicular nucleus, and the other has a small compact granular nucleus which is strongly basophilic. The cytoplasm of both types of cells seems to be very fluid, and the cell walls are difficult to distinguish. In the anterior end of the worm (Fig. 7) an invagination takes place to form the gut. This ingrowth seems to consist of an internal reorganisation of the cells. At this stage no specialisation of the cells lining the invagination could be ascertained. Behind this invagination no differentiations have occurred, and the caudal lamina has the same loose organisation of the two types of cells found in the rest of the body.

On the twenty-seventh to the twenty-ninth day after the egg was voided, the embryo becomes active inside the shell, and expansion and contraction of the caudal disc can be observed. This embryonic stage is similar to the newly emerged larva. If the shell is dissected away, the larva is active for only a short period and soon dies. The small and large hooks can be seen in motion, and in side view the small hooks appear raised above the level of the caudal lamina which has undergone considerable development. The small hooks are open to the exterior, a cuticular ring has been formed around each one, and the seven lateral pairs are placed on a slightly more developed shaft than was observable on the twenty-sixth day. The gut has differentiated posteriad and seems to end in a compact cellular mass. Sections of these stages are identical with those of the emerged larva and will be discussed later. On the twenty-eighth to the thirty-second day the operculum opens and the larvae emerge (Fig. 5). Either end of the larva may emerge first. If the anterior end leaves the egg first, the worm sometimes has trouble in freeing the caudal disc. When free, it may either swim by means of the caudal disc or creep by extending the body to almost twice the normal length, attaching the oral sucker, and then drawing up the caudal disc.

When they emerge the larvae are in various stages of development (Figs. 9, 10), but are approximately $0{\cdot}56 \times 0{\cdot}1$ mm. The body is of uniform width throughout, except in the region of the caudal lamina which is about 0·21 mm.

wide and 0·14–0·17 mm. long. It is the most highly differentiated and prominent feature of this stage, although it does not usually show the marked infolding at the posterior region which is characteristic of the adult condition. When creeping or swimming in the water, the larva tends to fold partially the two lateral edges toward the ventral side. The armature of the larva appears very formidable, and is disproportionately large when compared to that of the adult.

The small hooks of the caudal lamina (Figs. 10, 15) about 0·25 mm. long are arranged, as previously mentioned, with seven on each side and two in the centre. Around each of the two central hooks a thin flexible cuticular ring is formed which later develops into a sucker. Cross-sections through the ring show a concentration of cells in this region, a thickened cuticula, and a depression formed around each small hook. The small hooks of the caudal lamina can be moved rapidly and have been observed to revolve in a complete circle. In sections of larvae at this stage no muscle fibres could be found attached to the hooks. The large hooks (Fig. 12) are 0·11 mm. long, and are not as active as the smaller ones. A definite increase in the length of the shaft can be noted when it is compared with the twenty-six day stage (Fig. 11).

Except for the digestive tract, the histological structure has changed very little from the condition found on the twenty-sixth day. Considerable variation is shown in the development of the digestive tract (Figs. 9, 10) at the time of emerging. In certain of the larvae it consists of a mass of cells which will later develop into the pharynx (Fig. 6) and an invagination (Fig. 7) at the anterior end which ends blindly among the body cells. The cells which form the pharynx are probably differentiated from the large vesicular cells previously mentioned. In the larva, the pharynx is developed posterior to its normal position in the adult. In other larvae the digestive tract has invaginated to the pharynx, which has assumed a fibrous appearance, and formed a lumen opening posteriorly into a small round sac (Fig. 10). If a larva is observed for several hours a split can be seen to occur in the centre of the sac to form a ring which becomes the gut. The digestive tract is now lined by a uniform layer of cells which slightly resemble the large vesicular cells in the body. The excretory system is well developed at this stage, and the two large excretory vesicles and the large ducts, which run posteriad, can be clearly seen. The vesicles contract alternately.

The larva is very active for a few hours, and if it fails to find a host, becomes exhausted, sinks to the bottom, and dies. If it comes in contact with a necturus it becomes attached, migrates to the gills, and completes its development. No immature forms were ever observed on the skin. The gills furnish an abundant food supply and a well protected region in which the young worms can complete development.

Larvae were introduced into a tank of parasite-free necturi and recovered at various intervals. After five days of attachment to the host, a general development of the cells could be noted. The body of the worm is much more compact, considerable growth has taken place, and it is assuming the definitive

shape of the adult. The caudal lamina has undergone considerable development and the indentation characteristic of the adult condition is present. The large hooks have increased in size and are approximately 0·12–0·13 mm. long. The suckers have enlarged and are made up of fibres which are not as strongly developed as in the adult. The digestive system is now fully developed and extends to a point slightly above the anterior level of the caudal lamina. In sections it resembles the adult condition but is still somewhat smaller in size.

In larvae recovered ten days after attachment, the most significant change is the increase in the number of small granular cells between the intestinal branches in the middle half of the body. On the twelfth to fifteenth day, the most significant change is the development of testes, each with a definite limiting membrane. The cells which compose the testes are parietal cells, and are probably derived from the compact-nuclear type of cell previously localised in this area. Anterior to the testes, and on the left side, another localisation of cells occurs, which probably is the formative stage of the ovary. The area anterior to the testis is crowded with cells which may be the formative cells of the various genital ducts.

On the fifteenth to nineteenth day (Fig. 8) the ovary has appeared, and is composed of parietal cells, the vas deferens has formed, the parietal cells of the testes are actively dividing, and a suggestion of the female ducts is appearing. No genital pore could be distinguished. The large hooks at this stage are 0·16–0·19 mm. long.

Further development consists in the appearance of female ducts and the genital pore, increase in size of the entire organism, and development of mature spermatozoa and oogonia. These later processes could not be checked accurately on a time basis since they vary greatly in different specimens subjected to the same environment. Considerable variation is displayed in the larval development, and the time given for the stages mentioned above is the average from observations on a number of specimens. In general the life cycle is completed in a little less than two months.

### C. *Morphology of* Sphyranura polyorchis

The worms from Prof. H. W. Stunkard's collection are similar in shape to *S. osleri*. The type specimen (Fig. 2) is 2·4 mm. long. In width it is 0·77 mm. at the level of the intestine, 0·52 mm. in front of the caudal lamina, and 0·7 mm. at the caudal lamina. The caudal lamina is well developed with two suckers, two large and sixteen small hooks. The arrangement of the hooks and suckers is similar to that in *S. oligorchis*. No small spines could be observed at the curvature of the large hooks. The position of the muscles is the same as in *S. osleri*.

A strongly developed oral sucker (Fig. 2), diameter 0·3 mm., is subterminal in position. It has a limiting membrane and is sharply delimited from the parenchyma. Prepharynx present. The spherical pharynx is 0·15 mm. in diameter and the lumen extends from the anteroventral side dorsally and

posteriorly to open into the intestine. The intestine bifurcates in the region of the pharynx, and runs laterally and posteriorly on each side to the posterior end, where the two branches unite above the caudal lamina.

The male reproductive system (Fig. 2) consists of testes, vas deferens, seminal vesicle, and cirrus sac. There are from twenty to twenty-three testes which are irregular in size and shape and are crowded together in the middle half of the body (Fig. 2) between the branches of the intestinal tract. The testes are contiguous and form a double longitudinal series with occasionally three testes at the same level. The vas deferens passes from the region of the anterior testis, dorsal to the uterus, and ends in the cirrus sac. This cirrus sac has a coronet of eight spines which protrude into the genital atrium.

The parts of the female reproductive system which could be observed in the whole mounts consisted of a small ovary, oviduct, uterus, and vitellaria. The ovary is 0·085 × 0·065 mm., and has an oviduct leading from the posterior end. The uterus is large and capable of great distension. It opens into the genital atrium which in turn opens to the ventral surface by means of a genital pore and which is just behind the bifurcation of the gut. No seminal receptacles could be found in whole mounts or in sectioned material. Since the sections were poor, it is possible that vestigial seminal receptacles could not be demonstrated. The whole middle half of the worm, except the region where the testes are located, is obscured by the vitelline follicles (Fig. 2) which are arranged completely around the branches of the intestine. The vitelline follicles are filled with yolk droplets which give them a homogeneous yellow colour. One of the slides contained an immature form which looked very similar to a young specimen of *S. oligorchis* except for the number of testes and the appearance of the oral sucker.

## V. Discussion

### A. *Specific identity*

In the present study of specimens of *Sphyranura* from North America, two new forms have been found which appear to be separate and distinct species from *S. osleri*. In approaching the problem of whether the worms described as *S. oligorchis* and *S. polyorchis* are distinct species, three questions arise: (1) that they are alternate stages in the life cycle of *S. osleri*; (2) that they are precociously mature forms which will later develop the *S. osleri* condition; (3) that they are merely a variation produced by environmental conditions which affect the host and consequently the parasites. Since the life cycle in *S. oligorchis* has been repeatedly followed in detail from the time of hatching until death, and large numbers of worms have been observed over a period of more than twelve months, the first and second possibilities may be dismissed. The third question is more difficult to answer, since it is impossible to say positively that such a variation does not and could not arise in nature. It is

unlikely that such a variation does occur since we have obtained examples of only *S. oligorchis* in several different shipments of animals. The necturi when obtained were kept under controlled conditions which have approximated the normal habitat, and should not have produced or maintained variations in the parasite. Therefore, it appears that the worms described as *S. oligorchis* have a constant and definite form. A comparison of the specific characters of *S. osleri* (Wright & Macallum, 1887) with the specimens here reported shows clearly that they are a different species.

They differ from *S. osleri* in the following points. The number of testes reported for that species is 12–16, whereas *S. oligorchis* has from 5 to 7. *S. osleri* has a pair of functional seminal receptacles while *S. oligorchis* has two lateral pockets which are vestigial and show no evidence of a cavity. The ovary in the former is 0·100–0·160 mm., and in the latter about 0·065–0·075 mm. The vitellaria have an altogether different appearance from that figured by Wright & Macallum for *S. osleri*. In the worms studied, the vitelline follicles are not crowded together around the intestine, and do not have the solid homogeneous yellow appearance described and figured for *S. osleri*. In the original descriptions of *S. osleri* no mention was made of the presence of a densely packed area of cells located between the caudal suckers, as described for the present specimens. These cells take a characteristic stain and show clearly in whole mounts, although no definite function has been ascribed to them. On the basis of description and figures given for *S. osleri*, there seems to be a difference between the oral suckers of *S. osleri* and *S. oligorchis*. The oral sucker of the latter species, as shown in Fig. 1, is not quite characteristic, since it appears slightly more subterminal in living worms. The large hooks do not have the small spines of *S. osleri*.

In view of the differences between these worms, a second species, *S. oligorchis* Alvey (1933), was described with the following diagnostic characters:

*Sphyranura oligorchis*. Body 2·5–3·5 mm. long, 0·3–0·4 mm. wide; caudal lamina 0·7–0·82 mm. wide, and 0·35–0·47 mm. long; large hooks without spines; poorly developed oral sucker; ovary 0·065–0·075 mm.; vitelline follicles scattered; a heavily staining area of cells between the caudal suckers; seminal vesicle present, 5–7 testes; a pair of vestigial, non-functional seminal receptacles.

The worms obtained from Prof. H. W. Stunkard differ from *S. osleri* and *S. oligorchis* in the following points. They have a well developed oral sucker entirely different from the weakly developed sucker of the latter species, and presumably like that of the former species. The testes, although the arrangement is similar to that in *S. osleri*, are more numerous than in either *S. osleri* or *S. oligorchis*. The ovary is approximately one-half the size of the ovary in the former and about the same size as the one in the latter. The vitelline follicles are large and filled with many globules and are entirely unlike those in *S. oligorchis* but resemble those figured for *S. osleri*. No seminal receptacles could be seen in the whole mounts or sections and no spines are present on

the large hooks, which suggests a similarity to *S. oligorchis*. The presence of these specific characteristics in immature stages indicates that the worms are not a later stage in the development of *S. osleri*.

In view of the constant differences of these specimens from previously described forms, a third species, *S. polyorchis* n.sp., is recognised with the following diagnostic characters.

*Sphyranura polyorchis*. Body 2·4–3·0 mm. long, 0·41–0·77 mm. wide; caudal lamina 0·7–0·92 mm. wide, 0·36–0·49 mm. long; large hooks without spines; well-developed oral sucker; vitelline follicles compact and strongly developed; seminal vesicle present; 20–23 testes; seminal receptacles either vestigial or absent.

### B. *Phylogenetic and systematic position of the genus* Sphyranura

In his original description Wright (1879) placed *Sphyranura* in the Polystomidae on the basis of similarity in structure of the suckers, the size and shape of the egg, and the form and number of caudal hooks. The excretory vesicles should be added to this list. He pointed out that the resemblance of the larva of *Polystoma* to *Gyrodactylus* was very striking, and stated: "The suckers of *Polystoma* are not developed simultaneously and *Sphyranura* is a transition form, where the formation of these is restricted to one pair." Since *Sphyranura* appeared to be an intermediate form between the Gyrodactylidae and Polystomidae he suggested that they might all be included in a single family with *Gyrodactylus*, the type genus, since it was probably nearer the primitive form.

A comparison of *Sphyranura* with the other polystomes shows many differences, especially in general shape, number of caudal suckers, and the arrangement and structure of the genital apparatus. *Sphyranura* lacks the lateral vaginae of the polystomes but has paired lateral ducts with no external openings which, due to the findings on *S. oligorchis*, may not serve as seminal receptacles.

Stunkard (1917) and Fuhrmann (1928) agreed with Odhner's (1912) statement that the seminal receptacles of *Sphyranura* represent the remnants of paired vaginae. Stunkard stated: "I agree with Odhner that the seminal receptacles of *Sphyranura* are homologous to the paired vaginae of *Polystoma*, and that this furnishes a splendid example of the change whereby the terminal part of the uterus has assumed the copulatory function." He remarked also that the transfer of vaginal function to the uterus in the distomes may be due partially to the assumption of endoparasitic habits. If this view is to be accepted it is difficult to reconcile it to Wright's hypothesis that *Sphyranura* is an intermediate stage in the evolution of the polystomes. The evidence offered by a study of the vaginae of the polystomes and the seminal receptacles of *Sphyranura* would seem to indicate that the latter is the more highly specialised of the two groups. The polystomes have assumed a pseudo-endoparasitic habit by becoming parasites of the pharynx and the urinary bladder,

and might logically be expected to have a more advanced stage in the transference of the vaginal function to the uterus. Since the genital apparatus is one of the criteria for our present classification of the Monogenea, it is a strong point against the allocation of *Sphyranura* to the Polystomidae on the basis of its being an intermediate stage in the evolution of the polystomes.

This view is also substantiated by ontogenetic evidence. *Polystoma integerrimum* has a ciliated larval stage with eye spots and a caudal disc which lacks the large hooks. The earliest larval stage of *Sphyranura* does not possess cilia or eye spots, and has a complete set of hooks and a pair of developing suckers.

From an examination of slides of *Polystomoides* and *Oculotrema* lent to me from the private collection of Prof. Stunkard, it is evident that the histological picture of *Sphyranura* is very different. The former have a varied parenchymal structure exhibiting compact and vacuolated cells and a large amount of fibrous tissue, while the latter has a very definite cellular organisation. A far more significant feature is the presence of a vascular system in *Sphyranura* and its absence in *Polystomoides* and *Oculotrema*. Looss (1912) stated that he believed the vascular system to be a sound basis for classification. Willey (1930) has called attention to the regularity of its occurrence in the various species of the families for which it has been reported.

As pointed out, the genus *Sphyranura* differs in many points from the type genus, *Polystoma*, and it is doubtful whether it should be included in the same family. If it has sufficient differences to be separated from the Polystomidae, Poche's creation of the family Sphyranuridae should be recognised.

### C. *Phylogeny of all Monogenea*

Larvae are very important in tracing the phylogenetic relationships of the higher invertebrate animals and parasites where the adults have undergone considerable specialisation. An examination of the various life cycles of the Monogenea reported in the literature shows that a free-swimming stage is common to all of them, with the exception of *Gyrodactylus elegans*. In most of them the ciliated larval stage has four eye spots, a well developed gut, and small hooks. The large hooks may or may not be present. The cilia are usually arranged in three bands, but in *Diplozoon paradoxum* they are uniformly ciliated and in *Polystoma integerrimum* they are arranged in five bands. At a later stage, just after attachment, the larvae lose their cilia and develop powerful posterior organs for attachment.

The ancestral type of the Monogenea was probably a free-swimming form with two pairs of eye spots, a uniformly ciliated body, and a simple straight intestine. This in turn gave rise to a parasitic form which had hooks, an elongated body, a powerfully developed organ of attachment, and a simple digestive system. An examination of the various types of development reported for the Monogenea shows that the ciliated form of *Dactylogyrus* and

its adult form most nearly correspond to what appears to be the primitive ancestral type. The ciliated larva of *Dactylogyrus* has two pairs of eye spots, a pharynx, three ciliated bands, and fourteen small hooks. The adult form is very similar to any of the young attachment stages reported for species of the Monogenea.

*Gyrodactylus elegans*, which belongs in the same family as *Dactylogyrus*, has a polyembryonic development, lacks the ciliated larval stage, and emerges from the uterus in a condition ready for attachment. The viviparous polyembryonic condition is unique for the Monogenea, and may have arisen through the retention of the egg in the uterus, with the consequent reduction and disappearance of the vitellaria. An indication of a tendency toward the viviparous condition is manifested by *Polystoma oblongum* Wright (1879). Wright reported that the eggs had developed and the larvae had escaped from the shell and were free in the uterus before the egg was voided. The larva had no cilia, two eye spots, and a caudal disc. Since his observations were made on only two specimens the results are somewhat inconclusive. If such limited evidence may be used, this ovoviparous condition certainly appears to be a stage in the development of the true viviparous condition as found in *Gyrodactylus elegans*.

In the development of *Sphyranura oligorchis* the ciliated larval stage is missing and this omission is probably correlated with the advanced stage at which the worm leaves the egg. The caudal disc of *Sphyranura* which has just emerged from the egg can be homologised with the caudal disc of *Dactylogyrus*. The resemblance is especially striking in larvae of *Sphyranura* which do not have an indentation at the posterior region of the caudal lamina.

Because of lack of information concerning the morphology and development of many of the Monogenea, any conclusion as to their origin is at present highly speculative. Indications, as to the probable ancestor, seem to point to the Monopisthocotylea and of the genera in this suborder, *Dactylogyrus* seems to agree most nearly with the reported larval forms.

As more complete data on the group are obtained, a more primitive type than *Dactylogyrus* may be found. An examination of the life cycles of the Monogenea, Digenea, Mesozoa, and Turbellaria, shows a free swimming larval stage to be present sometime in their development. This planula type is probably the primitive ancestor of the entire group, and the various orders have arisen at different times. This hypothesis would derive the parasitic platyhelminthes from the primitive rhabdocoeles and place the polyclad Müller's larva as a later development.

## VI. SUMMARY

The description of *Sphyranura oligorchis* (Alvey, 1933) and *S. polyorchis* n.sp. is presented together with an account of the development of the former. Study of the life history of *S. oligorchis*, and a review of the literature on the

life cycles of the Monogenea, show that at a corresponding time in their development embryonic stages similar to *Dactylogyrus* are present. It is postulated that *Dactylogyrus* is near the primitive ancestral type of the Monogenea, and that all of the parasitic platyheminthes may have arisen from a common planula ancestor.

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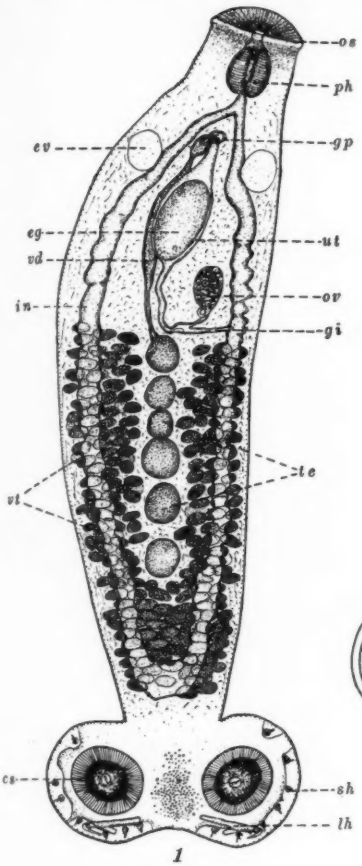
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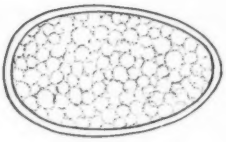

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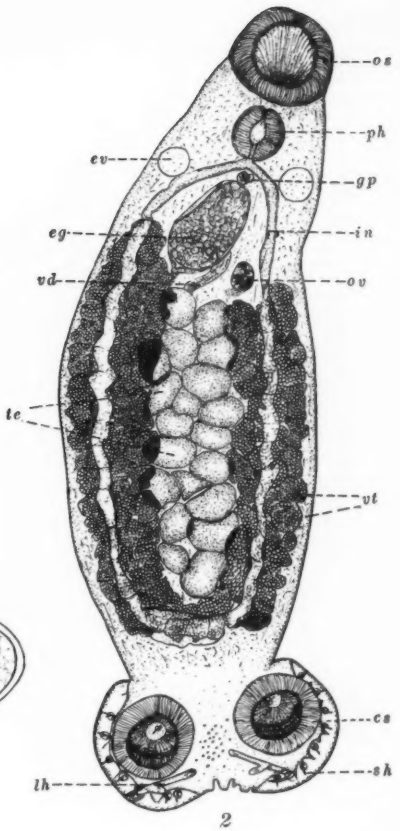
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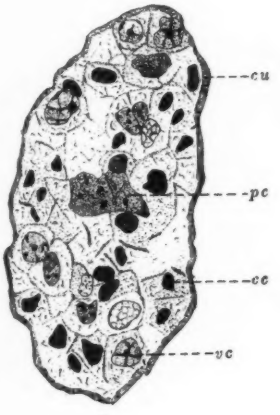
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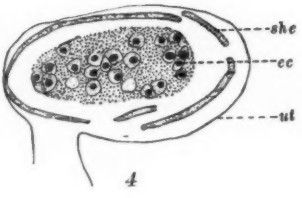
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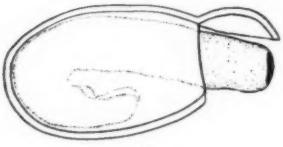

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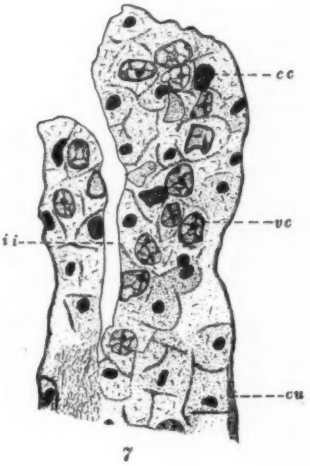
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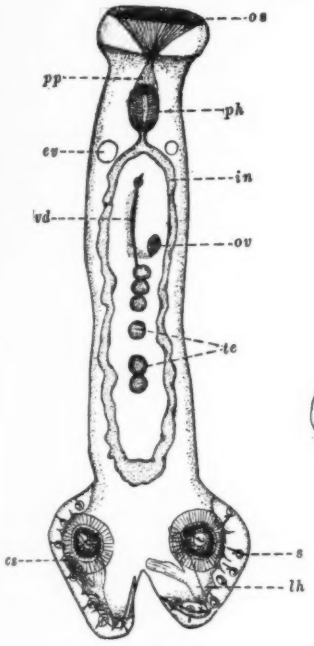


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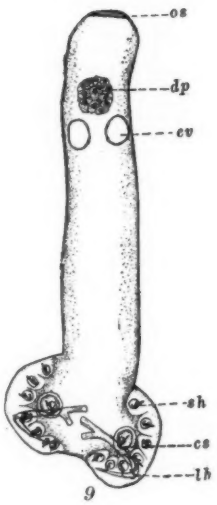


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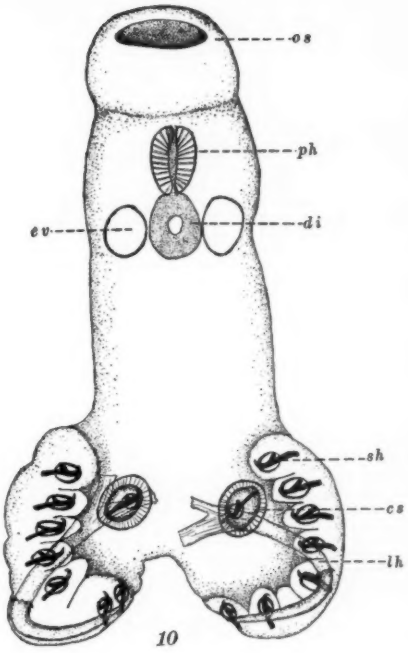


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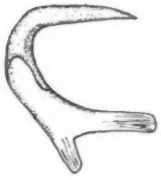

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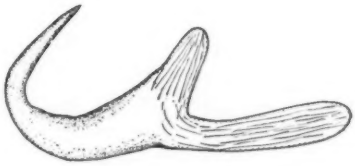

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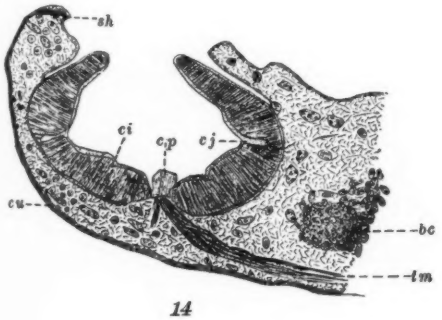


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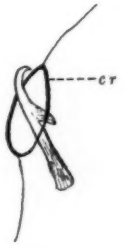


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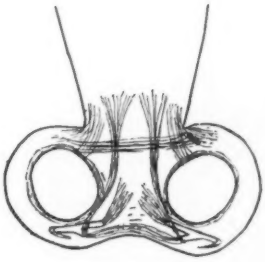

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## EXPLANATION OF PLATES

*bc*, basal cells of caudal lamina; *cc*, compactly staining cells; *ci*, cuticular indentation; *cj*, cuticular joint; *cr*, cuticular ring; *cp*, caudal plug; *cs*, caudal sucker; *cu*, cuticula; *di*, developing intestine; *dp*, developing pharynx; *ec*, embryonic cells; *eg*, egg; *ev*, excretory vesicle; *gi*, genito-intestinal canal; *gp*, genital pore; *ii*, invaginating intestinal opening; *in*, intestine; *lh*, large hook; *os*, oral sucker; *ov*, ovary; *pc*, pharyngeal cells; *ph*, pharynx; *pp*, prepharynx; *sh*, small hook; *she*, shell; *te*, testes; *tm*, transverse muscle; *ut*, uterus; *vc*, vesicular cells; *vd*, vas deferens; *vt*, vitellaria.

### PLATE X

Fig. 1. Type specimen, *Sphyranura oligorchis*, ventral view, ×58.
Fig. 2. *Sphyranura polyorchis*, ventral view, ×61.
Fig. 3. Egg, ×127.
Fig. 4. Cross-section of uterine egg.
Fig. 5. Egg hatching, ×127.
Fig. 6. Cross-section through larva which has just emerged from egg, camera lucida, ×760.
Fig. 7. Longitudinal section through anterior end of twenty-six day old embryo, camera lucida, ×760.

### PLATE XI

Fig. 8. Immature worm sixteen days after attaching, ventral view, ×66.
Fig. 9. Larva just emerged from egg, ventral view, ×188.
Fig. 10. Larva just emerged from egg, ventral view, ×262.
Fig. 11. Large hooks of twenty-six day embryo, camera lucida, ×181.
Fig. 12. Large hooks of larva, just emerged, ×181.
Fig. 13. Large hooks of mature worm, ×181.
Fig. 14. Cross-section through the caudal sucker, camera lucida, ×325.
Fig. 15. Small hook, ×680.
Fig. 16. Muscles of caudal lamina, ×127.

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# TURNING SICKNESS, A PROTOZOAN ENCEPHALITIS OF CATTLE IN UGANDA. ITS RELATIONSHIP WITH EAST COAST FEVER

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(With Plate XII)

## I. Introduction

From time to time outbreaks have occurred in different parts of Uganda of a peculiar disease of cattle characterised by symptoms indicating some lesion of the central nervous system. This disease is known to the natives of Uganda as "Kizengerera", or generally as "Turning sickness", which, besides being a literal translation of the native name, denotes the most remarkable symptom of the disease. At an early stage of our investigation into the pathology of "Kizengerera" we realised that we had to deal with a disease hitherto undescribed in veterinary medicine, and one of us (Mettam, 1934) published a very brief preliminary note in which attention was drawn to the most salient feature of the disease.

During the last few years we have been fortunate in obtaining a considerable amount of material for further research, and we are of the opinion that the time is now appropriate for the publication of a fuller account of the disease as it occurs in this part of Africa. Before commencing the paper, however, a very short survey will be made of the literature that can be found in various local publications referring to this disease, for, although the nature of turning sickness has only recently been elucidated, its presence has been known for many years. During the course of the present paper constant reference will be made to East Coast fever and the other theileriases. It is not intended to make any lengthy review of the literature relating to this group of diseases, since very complete bibliographies are given by Wenyon (1926) and Schilling & Meyer (1930).

It may be pointed out that the annual reports of the veterinary departments of the East African Territories contain useful and often valuable reports on investigations into the indigenous diseases, but it is often difficult for European workers to refer to these publications, which have a comparatively restricted circulation. Reviews of these papers are given, however, from time to time in the *Veterinary Bulletin* and should not escape the notice of those interested in East Coast fever and allied diseases.

## II. LITERATURE ON TURNING SICKNESS

Reference to the *Annual Reports of the Department of Agriculture, Kenya Colony*, shows that turning sickness, generally known in that part of East Africa by its native name "Muthiuko", has only twice been mentioned, namely in the reports for the years 1928 and 1933. In the former it is stated that five cases of the disease had been observed during the year in the settled areas of the colony, while in the 1933 report a heavy mortality is reported as being due to the disease in the Machakos district—Ukamba province—where it is said to have been responsible for more deaths than East Coast fever itself.

During a visit to Kenya in 1934 one of us (R. W. M. M.) saw a case of "Muthiuko" at Kabete and at once recognised its identity with "Kizengerera" of Uganda. The observation that "Muthiuko" is responsible for more deaths than East Coast fever must be noted. Such an assertion cannot be made in this country.

Up to the present the disease has not been reported from Tanganyika or the Congo.

As regards Uganda the disease was not officially notified before the opening of the Laboratory at Entebbe in 1930. In the departmental records at the veterinary office in Kampala, however, there is evidence that the disease attracted the attention of members of the field staff as far back as 1924, when Mr Fazal Din Ahmed, Indian Veterinary Assistant, made autopsies on several cases on a mission farm near Kampala. However, the nature of the disease was not recognised until 1930, when one of us (R. W. M. M.) demonstrated Koch's bodies in the brain of a case of the disease in the Mengo district. Since that year, brief accounts of our investigations have been published annually in the reports of the Uganda Veterinary Department, to which reference should be made by those interested.

In our preliminary communication (Mettam, 1934) the relationship between turning sickness and East Coast fever was briefly mentioned, but before passing on to a fuller description of turning sickness it is considered advisable to give a condensed account of our present knowledge of East Coast fever as it occurs in this country.

## III. EAST COAST FEVER AND THE THEILERIASES OF CATTLE IN UGANDA

### A. *General survey*

The presence of a fatal calf disease characterised by glandular hypertrophy has been recognised in Uganda for countless generations, and is one of the few diseases that the natives now claim was not introduced by Europeans.

This disease, which is known as "Amakebe", or simply as "Makebe", was first investigated by Bruce (1910) and his colleagues who primarily visited Uganda to work on Trypanosomiasis in man and the domestic animals. Subsequently, Theiler (1904) in the Transvaal, working with ticks collected off sick

calves of Uganda, confirmed that "Amakebe" was identical with East Coast fever of Southern Africa.

The indigenous cattle of Uganda possess a considerable resistance against this disease, and when the young sick calves are nursed the mortality may be as low as 10–25 per cent. Amongst the agricultural tribes, however, especially under adverse climatic conditions, the mortality is much more severe and up to 40 per cent. of the calves may succumb annually to this disease alone.

Within the last four years the tick-borne diseases of this country have been re-investigated, and from our experiments (shortly to be published) it would appear that in Uganda East Coast fever is a very fatal disease to imported or grade cattle, and even to native cattle from clean areas, but is of lower pathogenicity for indigenous animals, in which it may run a less acute or more prolonged course.

Recovery is regarded as being complete and breakdowns exceptional, and though the latter are from time to time recorded, no authentic cases have yet been investigated at this Laboratory. In South Africa, where East Coast fever has been investigated since the beginning of the century, it has been found impossible to cause relapses or breakdowns even after the most drastic procedures (du Toit, 1930), and the same holds good in Uganda.

There is no proof that any species of *Theileria*, other than *T. parva* or *T. mutans*, exist in Uganda, though a careful watch has been kept for their identification. It has been stated by Van Saceghem (1924) in the Congo that *T. parva* and *T. mutans* are the same species, but his experimental work is not convincing. From many observations made in this country, both in cattle exposed to ticks under natural conditions or to tick-feeding experiments under controlled conditions, it is quite certain that *T. mutans* and *T. parva* are immunologically and pathogenically different parasites. There is no proof, and certainly no indication, that *T. annulata* or *T. dispar*, as described by the French workers of Algiers or other observers in the Mediterranean region, exist in this part of Africa. The theileriases of Uganda, as also of Kenya and East Africa generally, resemble the theileriases of southern Africa more than those of northern Africa, which is not surprising, since there is indisputable evidence that these parasites were introduced into the Union of South Africa and Rhodesia in the early days of the present century by cattle from the east coast of the continent.

It seems from East African experience that the indigenous cattle, as the result of exposure to infected ticks over many centuries, have developed a variable degree of resistance which in some cases has become pronounced. That it is the local cattle that have acquired resistance and not the local parasite that has diminished in pathogenicity is clearly shown by the appalling losses experienced when highly susceptible animals are introduced into tropical Africa, or where an epizootic of the disease breaks out for the first time in hitherto clean parts of the continent, as in the West Nile district in 1921 and in the Congo (Schwetz, 1934). In localities in which the annual indigenous

losses are about 20 per cent. from East Coast fever the mortality amongst highly susceptible herds reaches 90 per cent. It cannot be too often emphasised that the acquisition of immunity by the stock in this part of Africa has proceeded far more rapidly than any decrease in virulence of the parasite as the result of repeated passage through cattle of ever-growing resistance. The disease as it occurs in Uganda may be briefly summarised as follows:

(1) A benign form in about 20 per cent. of cases. In the field these mild cases are often overlooked, but when temperatures of calves are taken daily from the time of birth the reaction is readily recognised. The lymphatic glands are never seriously affected; they slightly increase in size during the rise of temperature but never return to prefebrile dimensions. The mortality is negligible. During the reaction *Theileria parva* is very rare in the blood, while schizonts are only exceptionally found in spleen or lymphatic gland smears. This form of East Coast fever is seen both after natural exposure on the veldt and after experimental feeding of infected ticks. On recovery the calves seem to be solidly immune. Apparently this exceedingly mild form of the disease has been observed by Lichtenheld (quoted by Schilling & Meyer, 1930) and others in German East Africa, now Tanganyika Territory, and by Van Saceghem (1924) in the Congo (Ruanda-Urundi, and Kivu areas).

(2) The great majority of calves (80–90 per cent.) experience a clinical form of the disease accompanied by a rise of temperature, marked enlargement of lymphatic glands, general malaise, and respiratory distress. Parasites are usually numerous in the red cells, though on occasion even in these cases they may be far from common. Schizonts may be difficult to find in some cases, but as a rule they are present in large numbers. The mortality varies within wide limits depending on the way the sick animals are treated. If they are nursed and given plenty of milk it is low, but neglect, exposure to inclement weather, the presence of intercurrent diseases, unhygienic conditions and lack of milk increase the death-rate. Recovery is complete and a solid immunity is produced. No authentic cases of relapse have yet come to our notice, though a careful watch is kept for them at the Laboratory where the East Coast fever history of the young stock is known with certainty.

### B. *Pathology*

East Coast fever in Uganda shows some remarkable differences from the classical forms of the disease as described in southern Africa by Theiler (1904), Gonder (1911), K. F. Meyer (1909 and 1911) and Steck (1928); and in Kenya by Cowdry & Danks (1933). The lymphatic system is always involved but in some cases not severely. Renal infarcts, or "white foci", are very typical lesions in one or both kidneys in South Africa and Kenya, occurring in about 50 per cent. or more of the cases. In Kenya, Walker (1930) states that they are the most characteristic lesion of the disease. In Uganda they are much rarer, being observed in between 10 and 20 per cent. of post-mortem cases at the Laboratory. Apart from the lymphatic system, the lungs are most often

affected in Uganda, showing a variable degree, often marked, of oedema and congestion. The presence of abomasal lesions is inconstant and the degree of ulceration never so serious as in Kenya or South African cases.

These observations were made on experimental uncomplicated cases of the disease in indigenous calves. In imported stock and in native cattle from non-enzootic areas the pathological picture more closely approximates that described in southern Africa.

Very careful and special attention has been paid at Entebbe to the brains of animals dead from the disease. In the great majority of cases the nervous system is perfectly normal, but in some there may be a very moderate degree of meningeal stasis. No gross lesions within the actual brain tissue have ever been observed. Reference to the literature confirms that the central nervous system is rarely if ever involved in East Coast fever even in highly susceptible animals.

Steck (1928), working in Theiler's Laboratory near Pretoria, discusses in great detail the post-mortem of thirteen cattle destroyed in the last stages of the disease for collection of material. In twelve animals he reports that "the nervous system shows nothing unusual", and in one beast "the choroid plexus was bluish discoloured, otherwise nothing unusual". More recently in Kenya, Cowdry & Danks (1933) state that "both lesions and Koch's bodies are seldom found in the nervous system", and "we observed Koch's bodies in the cerebral cortex in only two out of twelve animals which showed them in other parts of the body".

It may be mentioned here—the point will be discussed again—that, at this Laboratory Koch's bodies have only been observed in the cortical smears of one case of East Coast fever, though the brains of twenty calves have been carefully examined.

There thus appears to be unanimity throughout eastern and southern Africa that, in genuine East Coast fever, the central nervous system is either free from pathological lesions, or only mildly affected. The same applies to the presence of schizonts or of Koch's bodies. If they are found they are extremely rare; as a rule none can be demonstrated at all.

## IV. Incidence

Turning sickness is well known to the cattle-keeping tribes throughout Uganda. It is particularly prevalent in Ankole, Lango, Teso and the Buganda provinces, where it is everywhere known as "Kizengerera".

The Bahima consider the disease to be infectious and water-borne. They maintain that losses are most serious during the hot dry seasons when large herds of nomadic cattle congregate at water holes. They do not believe that it is tick-borne nor that it has any connection with any other disease, including East Coast fever. Its fatal nature is well recognised by these observant cattlemen, who maintain that cows that have had two or three calves and stock from 1 to 1½ years old are the chief sufferers. Acute cases are said to be nearly

always fatal, but if an animal survives 10 days or longer the chances of complete recovery are regarded as being very good.

Treatment adopted by these people is very drastic and consists in free venesection and drenching with large doses of a herbal infusion in native beer. No success has attended requests for specimens of the plant used.

The Bahima opinion or theory of the cause and cure of this disease has spread all over the protectorate and is much credited even by European stockowners who largely employ men of this tribe as cattlemen or herdsmen.

The greatest number of cases, however, that has been investigated were obtained on the departmental experimental stock farm at Koja, Mengo province. Koja forms a peninsula which runs into Lake Victoria from the coast of Kyagwe, some 40 miles east of Kampala by road. The whole of this part of Buganda or Mengo is a highly enzootic East Coast fever area, and it is not unusual for calves to contract and recover from East Coast fever during the first 6 weeks of life. The mortality is very severe, and in some years 40 per cent. or more of the calves are lost from this disease alone.

The farm at Koja is subdivided into two main divisions, viz. (*a*) Luwafu, at the very extremity of the peninsula, and (*b*) Mpungwe, the main central portion of the estate. East Coast fever does not exist on Luwafu, and here are kept pure-bred and grade stock. Mpungwe, however, is a highly enzootic area and is grazed over by several different herds of cattle. On Mpungwe dipping is not carried out, but hand deticking is adopted, since one of the objects of the farm is to teach the natives the rudiments of animal husbandry without the assistance of a cattle dip, the cost of which would be prohibitive for the ordinary native cattle owner. The tick fauna of both Mpungwe and Luwafu has at different times been very carefully examined. The common vector of East Coast fever, *i.e. Rhipicephalus appendiculatus* (the brown tick), exists on both estates, but on Luwafu, as has been shown by suitable feeding experiments, it is not infected, and here East Coast fever has not been diagnosed. On Mpungwe, on the other hand, the brown tick must be infected to a considerable degree, since constant deticking, while reducing the incidence of ticks, has not reduced the morbidity rate of the disease. Other ticks collected include *Rhipicephalus bursa* and *R. evertsi* in rare numbers; *Boophilus decoloratus, Amblyomma variegatum,* and *A. cohaerens.* The first two are potential vectors of East Coast fever.

Examination of the Mpungwe records shows that turning sickness attacks cattle of both sexes and of all ages, from a couple of months to several years. Most cases occur in the hot dry season of the year, before the short November-December rains. On Luwafu, which is free from East Coast fever, the disease has never been observed. Elsewhere in Uganda the history is the same, all cases that develop the disease are born and bred in enzootic East Coast fever districts.

As mentioned in the introduction the same disease occurs in Kenya under the native name of "Muthiuko". Those who have seen cases of "Kizengerera"

in Uganda and "Muthiuko" in Kenya have no doubt about their identity. At the present time, however, the authors have no information on its distribution in Kenya, but they understand that the Kenya authorities are working on the subject. There appears to be plenty of evidence in both territories that the disease is commoner than is recognised.

No authentic cases have yet been reported from the mandated territory of Tanganyika since the British occupation, though several members of the veterinary department of that country believe that the disease is widespread. No record can be found as to whether the disease was recognised by the German authorities.

## V. The material

The individual history of the twenty-four animals that furnished the material for our experimental investigations into turning sickness will now be given in some detail.

Unfortunately serious blanks exist in certain of the histories, but these are quite unavoidable. It must be remembered in extenuation that Uganda is a tropical country in which distances are long and transport facilities far from perfect, so that it may be impossible to send living animals showing acute symptoms to the Laboratory for more detailed examination. Great credit is due to those members of this department, both European and African, who often at great trouble or inconvenience managed to supply us with suitable material. Whenever possible living cases were supplied, since they could be more systematically examined at the Laboratory and fresh tissues obtained for transmission purposes. In most animals, however, the ante-mortem histories, the post-mortem records and the histological material were supplied by field members of the staff including trained African assistants.

Where an animal was purchased from natives no reliable life history could be obtained, so that it is simply recorded whether or not it came from an East Coast fever enzootic area, information that could always be confirmed by the local district veterinary officer.

*Case I.* Eight months old Ankole-Zebu bullock born and bred at Entebbe, where it had an East Coast fever reaction when 2 months old. Subsequently it was dipped twice weekly. Four months later it developed nervous symptoms and was destroyed in a moribund condition after being ill for 8 days. Brain lesions were of acute haemorrhagic type, and numerous schizonts were found in the cerebrum.

*Case II.* A 3 years old bull purchased in Ankole—a highly enzootic area—for production of anti-rinderpest serum at Entebbe. Three weeks after arrival at the Laboratory—also an enzootic area—it developed acute nervous symptoms and was destroyed for collection of material. Brain lesions were similar to those seen in Case I. Numerous schizonts were found in smears of the cerebrum. Interval between East Coast fever and turning sickness is not known with certainty, but probably at least 2–2½ years.

*Case III.* This animal was a Zebu ox purchased in Kampala for research purposes. It had only recently come from the Eastern Province where East Coast fever is enzootic. Nothing is known about its early history. Shortly after its arrival at Entebbe it showed characteristic nervous symptoms and was kept under observation for 5 months. It was

destroyed *in extremis*. The left cerebral hemisphere was markedly atrophic, fibrous and a deep yellow colour. No schizonts were seen in brain smears. Interval between East Coast fever and turning sickness is not known with certainty.

*Case IV*. A 2½ years old bull. This animal was purchased in a district the greater part of which is enzootic, and sent to Entebbe by road—a distance of some 250 miles. Five days after its arrival at the Laboratory it developed nervous symptoms and died within 72 hours of the onset of the disease. The brain lesions were similar to those described in Cases I and II, but, in addition, there were symptoms suggestive of East Coast fever, viz.: (*a*) renal infarcts, (*b*) petechiae on the caecal and colic mucosae, (*c*) subacute abomasitis with ulceration of the mucous membrane, and (*d*) presence of numerous schizonts in the lymphatic system, liver, lungs, spleen, myocardium as well as in the brain. The absence of any definite history of a previous attack of East Coast fever is most unfortunate. Possibly the animal escaped the disease as a calf, or was bred in a "clean" kraal and contracted infection during the journey to Entebbe, or the stress of the long journey and the change from one enzootic area to another may have combined to cause a relapse, or again, it may represent a very rare type of turning sickness in which schizonts are numerous throughout the body.

*Case V*. An 8 months Zebu calf born and bred in a notoriously highly enzootic area. It had passed through an East Coast fever reaction some 7 months before the appearance of nervous symptoms. The animal was destroyed after being ill for a week and on autopsy haemorrhagic lesions were found in all parts of the brain in which numerous schizonts were also present. The rest of the carcase was healthy. The cerebro-spinal fluid of this animal was under considerable pressure, but no parasites could be demonstrated in it.

*Case VI*. A 6 years old milch cow, the property of King's College, Budo, near Kampala. The animal lived in an enzootic area until 6 months old, and there is a definite history of its having had East Coast fever. For the next 5 years it lived on a European owned estate, was dipped twice weekly, and remained in excellent condition. Six months before death it was returned to Budo where it was neither dipped nor deticked by hand, and consequently the tick infestation became incredibly severe. No symptoms indicating East Coast fever were recognised during the last 6 months of life. A week before death very marked nervous symptoms appeared and the animal was destroyed while *in extremis*. On post-mortem, very extensive haemorrhages were found in the cortex, corpus striatum, hippocampus, mid-brain and cerebellum. The rest of the body was healthy. Schizonts were very numerous in brain smears. The great interest of this case is that such pronounced brain lesions appeared within 6 months of the animal being re-exposed to intense infestation by potentially infective ticks. It is recognised that after being dipped for so many years its immunity to East Coast fever must have greatly declined, but no symptoms of this disease were apparent during the last illness. Possibly the nervous symptoms were due to schizogony occurring in the brain in a partially immune animal. This possibility will be discussed more fully later.

*Case VII*. Six months old heifer, No. 604. Born and bred in a highly enzootic area, it had a mild attack of East Coast fever when 2 months old, from which it apparently made a complete recovery. Its final illness lasted 4 days and nervous symptoms were very pronounced. Schizonts were confined to the brain. Interval between turning sickness and East Coast fever was 6 months.

*Case VIII*. Eight months old bull calf, No. 639. Its history is very similar to the preceding. It had a definite history of East Coast fever during early life and recovery appeared to be complete. After 6 months typical nervous symptoms appeared and the animal was destroyed 5 days later when moribund. The brain showed extensive haemorrhagic lesions in which schizonts were very numerous. The lymphatic system, spleen, liver, and kidney were normal and showed no schizonts.

*Case IX*. A 14 months old bullock, No. 656. Five months before developing turning sickness it had East Coast fever. The animal was born and reared in a highly enzootic area. Nervous symptoms were peracute and the animal died within 48 hours. Very acute haemor-

rhagic lesions were present in all parts of the cerebrum in which schizonts could be readily demonstrated. All other organs were quite normal. Interval between the appearance of nervous symptoms and East Coast fever was 5 months.

*Case X.* Four years old bull, No. 570. There was definite history of East Coast fever when 6 months old, but for the next 3½ years the animal lived in a highly enzootic area without apparent ill effects. During the last fortnight of life the animal was "off colour", but nervous symptoms were not very pronounced, though it showed a peculiar staggering gait with swinging of the hind-quarters. On post-mortem there were petechial haemorrhages in the vicinity of the lateral ventricles. Schizonts were very numerous.

*Case XI.* Heifer of dam No. 508. This calf was born and bred in a highly enzootic area and was infected with East Coast fever when 2 months old. Four months later it showed nervous symptoms and died within 48 hours. Numerous schizonts were found in the brain, but none could be demonstrated in the spleen or lymphatic glands.

*Case XII.* Bull calf of cow No. 421. As in the case of the previous four animals, this calf was exposed to infected *Rhipicephalus* ticks from the date of birth. There is no history of its ever having had clinical East Coast fever, but it was only 5 months old when it developed nervous symptoms. It must be remembered, however, that in highly enzootic areas many calves react to East Coast fever without showing any clinical symptoms beyond a very transient febrile reaction. Such cases, unless daily temperatures are taken during the first year of life, are overlooked, and when experimentally exposed to East Coast fever fail to show signs of illness. It is certain that all susceptible calves in the presence of massive infestation by infected ticks pass through East Coast fever, but the severity of the disease varies from an acute fulminating fatal attack to a mere febrile reaction. At post-mortem, the cerebellum, medulla and mid-brain of this calf showed haemorrhagic foci in which Koch's bodies could be readily demonstrated.

*Case XIII.* Heifer No. 620. This animal was born in the same locality as Case XII. When 4 months old it suffered from East Coast fever and apparently completely recovered. Nineteen months later it developed mild nervous symptoms. The animal was taken by car to the Laboratory where it was kept under observation for a month, during which the symptoms became milder and eventually gradually but completely disappeared. While at Entebbe its resistance to East Coast fever was tested by exposure to infected ticks, but its immunity proved to be solid. The animal is still alive and has so far shown no recurrence of the brain symptoms.

*Case XIV.* This case is of interest in that it occurred in the Lango district of the Northern Province, thus affording definite evidence of the existence of the disease in that part of the colony. No history of East Coast fever was obtained, but the subject—an aged cow—had been born and bred on one of the most highly enzootic areas in the country. The animal showed very acute nervous symptoms and died within 36 hours of being reported ill. On autopsy, extensive haemorrhagic lesions were found in the cerebellum in which numerous Koch's bodies were found on histological examination.

*Case XV.* A 9 months old calf (No. 535) born and bred on an enzootic East Coast fever area. As in the case of No. XII this animal had no apparent clinical East Coast fever. The animal showed nervous symptoms for 6 days before death. At post-mortem, haemorrhages were scattered through the brain and schizonts were present in great numbers in this organ, though none could be detected in the spleen or lymphatic system.

*Case XVI.* Kabete case (Kenya). This animal—a yearling heifer—was seen by one of us (R. W. M. M.) while on a visit to the Veterinary Laboratory, Kabete, Kenya Colony. The animal was born and bred in a highly enzootic East Coast fever part of the colony, and Mr Daubney is of the opinion that it could not have escaped having had East Coast fever during early life. The heifer was sent to the Laboratory, Kabete, where the similarity of its history and symptoms to the turning sickness of Uganda were recognised. Mr Daubney destroyed the animal, which was in a moribund condition. The brain showed very extensive

haemorrhagic lesions in which Koch's bodies were found, though not in large numbers. Two schizonts were seen in the spleen but none in the gland after prolonged search. The animal was ill for about 23 days.

*Case XVII.* Calf No. 504. This animal was born and bred in an enzootic area, and there is definite history of its having had East Coast fever when only 2 months old. Apparently it recovered completely from this disease, but 10 weeks later showed nervous symptoms from which it died after being ill for 72 hours. Very large haemorrhages were scattered throughout the left cerebral hemisphere. Schizonts were confined to the brain lesions.

*Case XVIII.* Calf No. 601. This calf came from the same estate as the preceding animal, and it is definitely known to have had East Coast fever. Exactly 3 months later it developed nervous symptoms. Unfortunately the brain was not submitted for examination, but smears from the cortex showed numerous schizonts while none was found in the gland or spleen. The blood showed a severe infection of *Theileria*.

*Case XIX.* Yearling heifer calf No. 706, born and bred in an enzootic area. Its early history is quite unknown. When admitted at the Laboratory it was very emaciated and quite blind, but no nervous symptoms were observed. It was destroyed in a moribund condition. Lesions were confined to the optic thalamus and left hippocampus in which there were numerous small cavities containing a yellowish fluid. Both structures were a deep lemon colour, atrophied and of a distinct fibrous nature. Schizonts were absent in hippocampus, optic thalamus, cortex and all the visceral organs. A severe infection of *Theileria* was present in the blood.

*Case XX.* A 15 months old calf. Born in an enzootic area within 6½ weeks it contracted a severe form of East Coast fever. Subsequently she recovered but remained "stunted" in growth and in very poor condition. Twelve months afterwards she developed nervous symptoms and died within 48 hours. Schizonts were numerous in the brain but apparently absent from the spleen and lymphatic system.

*Case XXI.* A 3 years 10 months old grade bull No. 34. Born and bred in an area free from East Coast fever, it lived for 17 months before being transferred to a fenced paddock which had been cleared of ticks but was surrounded by enzootic pasturage. Here it never showed any clinical symptoms of East Coast fever. However, 16 months later it was moved to a different paddock in which were numerous infected ticks. Within 30 days it developed acute nervous symptoms and died after 4 days' illness. Unfortunately there is no detailed report on the post-mortem of this animal, but stained films showed numerous Koch's bodies in the brain and in the lymphocytes of the peripheral blood. Very rare schizonts were found in the lymphatic system, but none at all in the other viscera.

*Case XXII.* Bull calf No. 1534. Born and bred on a farm in an enzootic area, and when 7 weeks old showed an East Coast fever reaction from which it apparently completely recovered. Seven and a half months later it developed nervous symptoms in a subacute form and was sent to the Laboratory where it was destroyed. Schizonts were numerous in the brain, but the lymphatic system and blood showed no parasites.

*Case XXIII.* Bull calf No. 560. Born and bred in an enzootic area. Shortly after birth it had a very mild attack of East Coast fever, and 8 months afterwards showed acute nervous symptoms. Schizonts were confined to the brain.

*Case XXIV.* Heifer calf, unnumbered. Early history quite unknown. When 12 months old it developed acute nervous symptoms, and on post-mortem showed numerous Koch's bodies in the brain. All other organs were perfectly healthy and apparently free from parasites.

## VI. SYMPTOMS

May be acute or chronic. The latter may result from an acute attack or be chronic in nature from the beginning.

(*a*) In acute cases the onset is very sudden. Animals, hitherto regarded

as perfectly healthy, develop violent nervous symptoms such as spinning or turning in one direction until dizzy, when they collapse on to the ground. At this stage the animal is either comatose or unconscious, while salivation may be very profuse. During the paroxysms individual muscles or large groups of muscles may twitch or tremble, especially the muscles of the flank, gluteal regions and those covering the shoulders and sides of the neck.

While on the ground the animal may struggle slightly or show galloping movements of the limbs.

Consciousness slowly returns and within a few minutes the animal rises to its feet and may actually nibble at the grass. In peracute cases the paroxysms may last 3 min. or longer; in most animals they are considerably shorter. It is difficult to say how often these fits occur. In severe cases they become more and more intense and appear at shortening intervals until death occurs from exhaustion. In other animals they tend to occur less frequently and to be milder.

If the animal is examined during a violent paroxysm it will be noted that the respirations are hurried, the muscles of the nostrils quiver or twitch, and the pulse is very weak or quite imperceptible. The eyelids are wide open showing an injected conjunctiva and greatly dilated pupils. The animal has a maniacal or frenzied appearance.

During actual turning there is very remarkable lack of co-ordination resulting in the animal treading on its own feet, or rolling and lurching in the most drunken manner.

There is sometimes a very noticeable localised hyperaesthesia as shown by complete absence of response to pin pricks.

Very often the animal remains greatly depressed between the fits and may adopt and remain in awkward unnatural postures. A very noteworthy feature of the disease is the absence of any fever, the temperature being normal in most cases, though a rise of one degree or so has been reported. It is certain, however, that the condition is not a febrile one.

In less acute cases all the above symptoms are milder. The turning movements are not so rapid and the depression is not so great. The animal tends to stand apart from the rest of the herd and may be seen standing with its head depressed and pushed against a tree, fencing post, or even an ant-hill. Symptoms are frequently exaggerated by external stimuli, such as the approach of man or other animals, or if the animal is forced to move. Appetite and rumination appear to be only seriously impaired during the paroxysms. The superficial lymphatic glands are not noticeably altered in size.

At times, even in acute cases, the animal may appear to be completely blind. This condition may only be temporary and last a short time, or it may be permanent. Undoubtedly its permanence depends on whether the visual tracts in the brain have been irreparably damaged by the haemorrhages. In chronic cases corneal and/or lenticular opacities are usually present. Rarely, there may be no apparent lesions in the eyeball.

Diarrhoea has not been recorded, though in some of the acute cases there was a very obstinate constipation. Difficulty in micturation was sometimes noticed, due apparently to the animal being unable to assume the correct position for voiding the bladder.

Although nearly all the above symptoms have been recorded in many acute cases, in others they are not so apparent and may actually escape detection. Several animals were found dead and the disease only recognised at post-mortem. There were very severe cases in which the pathological lesions were of an extensive apoplectic nature and responsible for much destruction of the vital areas of the brain.

(*b*) In chronic cases the animal appears unthrifty or is in poor condition. Generally they are quite blind in one or both eyes as the result of corneal or lenticular opacities or both, but in quite a number of cases there are no apparent intraocular lesions to explain the loss of vision.

Turning movements are never severe, violent or continuous. Remarkably few systematic disturbances may be recorded beyond loss of condition and impairment of vision. Generally, however, the animal lags behind the main herd, and when driven staggers and stumbles as the result of muscular incoordination. The forelimbs may actually cross one another, while the hind ones are dragged along the ground. Atrophy of certain muscle groups has been reported.

Most of these protracted cases die from exhaustion; only a few succumb during a paroxysm. Complications have not been observed beyond accidents to limbs as result of falling into pits, etc.

Death may be quite unexpected, but normally the final stages are very protracted.

*Course.* In six cases the illness terminated fatally within 48 hours, while four animals survived 4 days, and two animals 6 and 8 days respectively. In another two cases symptoms persisted for 14 and 25 days; both terminated in death. In three animals symptoms occurred periodically for 5, 5 and 6 months, but all died.

In seven animals the exact duration of the disease was unknown.

*Mortality.* It is not possible to state definitely the percentage of deaths, but to date no permanent recovery by any animal showing typical acute symptoms has come to our notice. Reported recoveries proved on further investigation to be temporary remissions of symptoms.

The majority of chronic cases also succumb to the disease itself, but some, the minority, are destroyed by their owners as being valueless, mainly because of incurable blindness, and few die as a result of accidents incidental to the disease, *e.g.* broken legs, etc.

*Diagnosis.* The occurrence of characteristic nervous symptoms unaccompanied by fever allow of a diagnosis of turning sickness, which will be readily confirmed on post-mortem examination. In those cases, however, in which symptoms are milder it is not always easy to make a diagnosis, and the real nature of the disease may only be recognised at autopsy.

Blindness is a valuable symptom for the detection of chronic cases, but it cannot be regarded as pathognomonic of turning sickness, since it may occur after certain other diseases particularly contagious ophthalmia. In every instance the clinical history of the animals should be ascertained.

In considering the diagnosis of this disease, it must be remembered that certain poisonous plants also cause nervous symptoms, in particular the plant *Matricaria nigellaefolia* (Compositae), which Andrews (1923) proved to be the cause of "Staggers" or "pushing disease" of cattle in Natal.

It has been shown by Theiler and others in South Africa that certain plants of the genus *Senecio* are toxic for cattle, producing symptoms of frenzy, muscular inco-ordination and staggering. The primary lesions are found in the liver and intestinal tract, the brain being only slightly involved. There should be no difficulty therefore in distinguishing *Senecio* poisoning from turning sickness. Further, none of the South African species have so far been collected in Uganda, where the commonest species is *S. abyssinicum*, whose toxicological history is known (Mettam, 1935).

Probably the most prevalent disease of cattle in tropical Africa, in which nervous symptoms appear, is heartwater. This is a tick-borne disease and a very fatal one to susceptible animals. Although the distribution of the bont tick (*Amblyomma variegatum*) does not exactly coincide with that of the brown tick, yet in most parts of Uganda the two ticks exist under the same conditions in great numbers, so that it would not be unexpected to find both turning sickness and heartwater similarly distributed. Heartwater has not yet been satisfactorily diagnosed in this country, but its presence is suspected and may be definitely proved at any time.

There should, however, be no difficulty in distinguishing between heartwater and turning sickness. The former is an acute febrile disease more pathogenic for sheep and goats than for cattle, while turning sickness only occurs in cattle. In heartwater chopping or masticating movements of the jaw and the alternate protrusion and retraction of the tongue are very characteristic symptoms which have never been observed in turning sickness. Bellowing and great nervous excitement are symptoms invariably observed in bovine heartwater in Kenya. In turning sickness these have not been observed; on the contrary the animal appears stupefied and greatly depressed. Symptoms common to both diseases, however, are muscular tremors, wandering in circles, pushing into objects, and in the final stages muscular spasms. Pathologically the diseases can be readily distinguished. The gross lesions observed in brains of turning sickness cases have never been found in the brains of animals dead of heartwater.

Koch's bodies can be easily demonstrated in smears of the brain in turning sickness, while heartwater may be readily diagnosed by the presence of *Rickettsia* in the endothelial cells of the tunica intima of any of the larger veins.

Experimentally, heartwater can be artificially produced in susceptible animals by the inoculation of virulent blood taken from a sick or recently

recovered animal (Galloway, 1930). As will be described later it has been found impossible to transmit turning sickness by the usual experimental methods. Where heartwater is suspected all doubts will be removed after blood inoculation.

Finally, turning sickness must be distinguished from the condition described by Carpano (1932) in which various immature forms of *Trypanosoma theileri* occur in the brain, causing symptoms of great depression and suppression of all voluntary movements. If the nervous symptoms of turning sickness already described in this paper should occur, they afford a ready means of differentiation from Carpano's disease; but where they are absent it may only be possible to make a final diagnosis by parasitological examination of brain material at post-mortem. At present there is no evidence that Carpano's disease occurs in Uganda.

## VII. PATHOLOGY

### *The central nervous system*

The presence of brain lesions may be noticed while removing the bones of the cranial vault. The meningeal vessels are observed to be deeply engorged with blood generally over the entire organ, more rarely only locally. Petechiae are often present on the dura mater, and large clots up to the size of a florin may be found in the meninges following ante-mortem rupture of some of the vessels. The venous sinuses in particular are greatly distended.

On removal of the dura mater the surface of the brain, in acute cases, has a deep pink or red colour due to the prominence of the pial vessels and numerous petechiae in the pallium. If the main pial strands running into the brain tissue are examined, the great engorgement of the vessels will be readily seen. The tela choroidea and choroid plexuses of the ventricles are swollen, soft and velvety to the touch, and a deep red colour.

In chronic cases the meninges are usually free from naked eye changes.

As regards the brain itself very remarkable lesions are found. In acute cases they are of the haemorrhagic type; in the chronic cases necrosis, atrophy, fibrosis and cavity formation are found. Naturally, the lesions vary considerably in size, severity and distribution. As a rule they are multiple and often cause serious disorganisation and actual disintegration of the nervous tissue.

Lesions are most frequently observed in the white matter (corpus medullare) of one or both cerebral hemispheres. They may only be found in this position, but generally they have a wider distribution, being present in such structures as the corpus striatum, hippocampus and olfactory lobes. The thalamic region and corpora quadragemina are less frequently affected, but in no animal were serious lesions observed in the medulla or pons. In one animal (Case XIV) haemorrhages were only found in the cerebellum, but as a rule this part of the brain is much less severely affected than the structures of the telencephalon.

As mentioned before, the lesions in acute cases are in the form of haemorrhages. The great majority exist as small punctiform or lenticular-shaped petechiae usually discrete but often aggregated into groups or clusters. They vary greatly in number, in some cases they are so numerous as to suggest that the brain has been sprinkled with blood. Small extravasations up to the size of a pea are not uncommonly observed. They are irregular in shape and cause laceration of adjacent brain tissues.

Considerably larger haemorrhages were observed in Cases I and XVI. In the former there was an extravasation measuring $4 \times 2{\cdot}75$ cm. and, in the latter, $5 \times 3$ cm. Both were found in the corpus medullare of the cerebrum. They were bright red in colour, irregular oval in shape, with a very uneven sinuous periphery. In the centre of each lesion lacerated and contused brain tissue greyish in colour could be made out. About the main lesion were numerous secondary haemorrhagic foci, discrete or collected in small clusters. The brain tissue in the immediate vicinity of these large extravasations appeared to be softer, more friable, and moister than the normal.

In Case IX the petechiae were particularly numerous in the hippocampus and corpus striatum, while free blood was actually present in the lateral ventricles.

The cerebrospinal fluid was only examined in a few cases since the great majority of the brains were removed by natives in the field. Under more suitable conditions at the Laboratory, however, it was found that in acute cases the fluid is often under considerable pressure and greatly increased in amount. It was crystal clear but after being centrifuged showed no cellular increase and was apparently free from parasites.

In chronic cases the brain is free from haemorrhages but shows lesions indicative of degenerative changes. Very often, certain parts of one or both hemispheres or even one entire hemisphere itself, as in Case III, was shrunken or atrophied.

A local or diffuse pale yellow discoloration is also a constant feature of these chronic cases. Probably this is due to the presence of pigment derived from the haemoglobin of the haemorrhages which occurred during the early stages of the disease, the haemorrhages themselves having been removed by phagocytosis. A similar discoloration of the brain has been described by Carpano (1932) in cerebral trypanosomiasis due to *T. theileri*.

Cavities containing pale citron-coloured fluid are typical of chronic cases. They are small, up to the size of an orange pip, and either single or multiple. They are found in all parts of the hemispheres, mid- and inter-brain. In Case XIX they were found on the ventricular surface of the hippocampus giving that structure a yellow finely reticulated appearance. In Case III they were particularly numerous in the corpus striatum and, especially, the optic thalamus, seriously disorganising these bodies and probably responsible for the complete blindness shown by the animal for several months before death.

The shrunken condition of the brain was very well illustrated in Case III,

in which the whole of the cerebrum was much reduced in size. On section it was markedly fibrous, rivalling a cirrhotic liver in consistence. In Case XIX the right hippocampus and pyriform lobe were considerably atrophied, the thickness of their walls being reduced to a mere shell or rind.

*Changes in other organs*

Most of the cases of turning sickness died some distance from the Laboratory, and it was not always possible to carry out detailed post-mortems. The brain was removed intact whenever possible and preserved in 10 per cent. formal saline solution, but, as a rule, only a cursory examination was made of the rest of the carcase. As mentioned previously most of the pathological material was collected by natives, some of whom were untrained and not competent to carry out careful post-mortems.

However, a complete examination of the entire carcase was made by competent observers in eleven animals, most of which were destroyed in a moribund condition for the collection of histological material.

There are no constant lesions in the thoracic or abdominal viscera of animals which have died from turning sickness. When any lesions are present it is doubtful whether they have more than a fortuitous connection with turning sickness but further investigation is required to clear up the point.

In three animals, Cases I, II and V, the carcase was perfectly healthy, but in four animals, Cases III, IV, XVI and XXIII, renal lesions were observed which in their pathological histology resembled either fairly recent (Case IV) or old standing East Coast fever lesions (Cases III, XVI, and XXIII).

This observation is of considerable interest, since it is known, at least in Cases XVI and XXIII, that the animals had had and apparently completely recovered from East Coast fever some 9 months previously, but there is no doubt that the lesions of both cases were slowly regressing and not of the active nature one would suppose them to be if they were primarily connected with such an acute disease as turning sickness.

Ulceration of the abomasum—a constant lesion of East Coast fever—was observed in three animals, namely Cases III, IV and XVI. Except in Case IV the ulcers were small, varied in number, and were of some chronicity as shown by the absence of any acute inflammatory reaction and by the lesions being in an advanced stage of cicatrisation. In Case IV ulceration was very mild but obviously of more recent origin. As mentioned previously, when the history of this animal was more fully described, there is some doubt whether this is a true case of turning sickness despite the presence of schizonts in the brain.

The spleen, lymphatic system, liver and lungs showed no constant or noteworthy features. In all eleven cases the glands were free from obvious lesions, a very important difference from East Coast fever, in which these organs are generally very extensively involved.

Petechiae were found on the endocardium in four animals, viz. Cases VI, VIII, XVI and XXIII. The haemorrhages were only numerous in one animal

(Case VI) and as a rule were small and scattered except in Case XXIII where they were more of the nature of subserous extravasations. It is noteworthy that all these four animals were killed for collection of material. The nature of these haemorrhages suggests a recent origin, independent of any previous East Coast fever reaction, which is known to have occurred in all four animals between 5½ and 9 months previously.

Despite the enormous amount of work done on East Coast fever during the past 35 years in different parts of Africa, nothing is known about the mechanism of recovery from the disease. As Cowdry & Danks (1935) stated "the tissue reactions leading to recovery remain a closed book".

It is not possible to say how long the so-called renal "infarcts" or the gastric ulcers persist before completely healing. It is, of course, known that such lesions are not permanent, for in the great majority of animals recovered from East Coast fever none can be found, showing that repair must proceed rapidly and regularly.

Whether delayed healing has any connection with turning sickness is a moot point, but it must be emphasised that in the majority of cases there is no constant lesion in any organ other than the brain.

## VIII. The parasite

If films are made from the cerebral haemorrhages or from the larger extravasations and stained by Giemsa, bodies which are of an undoubted protozoan nature are found, often in very large numbers. Closer examination shows that these structures are morphologically indistinguishable from the well-known Koch's granules or Koch's blue bodies which are present in East Coast fever and other diseases due to parasites of the genus *Theileria*. Koch's bodies occur in the theileriases in such large numbers that they are conveniently used for diagnostic purposes. They have been very extensively studied, and it is now accepted that they represent the schizogonous stage in the development of *Theileria* in the mammalian host.

The discovery of these bodies in large numbers in the brain lesions of animals dead of turning sickness has not previously been recorded. In East Coast fever or, as far as can be determined from the available literature, in the other theileriases, it is exceptional to find schizonts in this organ, and then only in rare numbers.

The schizonts of turning sickness are very similar in appearance to the schizonts of East Coast fever. They agree in size, structure and staining reactions. Both agamogonous and gamogonous forms are present. In all our cases the former predominated. The majority are intracellular even in films.

In certain brains (*e.g.* of Cases V, VI, X, XV, XVIII and XX) they were exceedingly numerous, far more so than the number of schizonts found in the spleen or lymphatic system in the majority of cases of East Coast fever in this country. It was not exceptional to find as many as 9 per microscopic field (1/12 oil immersion and 7 eyepiece).

In size they averaged between 5 and 10 microns, but many huge agamonts were seen from time to time. The nuclei stained characteristically with Giemsa and clearly showed the structural differences which have enabled various workers to divide the schizogonous stage into the two separate phases designated agamogonous and gamogonous respectively.

Considerable attention has been paid to the structure of these bodies with the object of finding whether they differ in any respect from the descriptions of the Koch's bodies of East Coast fever as given by Gonder (1911), Meyer (1911), Cowdry & Danks (1933) and others. The bodies appear to be identical in every respect, and there seems to be no doubt that the structures found in the brains of turning sickness animals are genuine schizonts. The persistence of the schizonts in the brain is remarkable, and there is evidence that they can exist for as long as 3 weeks after the onset of illness. In more chronic cases in which the haemorrhages have been absorbed and the brain shows extensive atrophy, fibrosis, or necrosis, they can no longer be found (Cases III and XIX).

In every brain examined the number of agamonts exceeded the gamonts, often by a large margin. Thus, in Case XVIII the relative percentages were 66·4–33·6; in Case X 76–24; in Case V 60–40; in Case VI 59·8–40·2; and in Case VIII 74·4–25·6. Similar figures were found in other cases examined. Now, as the case records show, these animals lived for a varying period after the onset of nervous symptoms. Case XVIII lived 3 days, Cases V and VI 2 days each, Case VIII for 5 days, Case XV for 6 days, and Case X for 14 days. Finally, Case XVI, which was ill for 23 days, showed schizonts in its brain, though not in large numbers.

As a rule, however, the schizonts were most frequent in those animals which died within a few days of the appearance of clinical symptoms and tended to become more and more scarce as the period of illness lengthened. It is not yet possible to state how long these bodies can persist in the brain. It is known that they are not found in old chronic cases. There is evidence to support the view that they can be found as long as the lesions are of a haemorrhagic nature, but that they disappear when retrogressive or phagocytic changes occur in or about the haemorrhages.

Even in East Coast fever, probably the most exhaustively studied of all the theileriases, absolutely nothing is known about the time required for the differentiation of agamonts into gamonts. Cowdry & Danks (1933) believe that the time is longer as well as shorter than 2 days. It may only be a few hours. It is impossible to state whether the agamonts in cases of turning sickness can survive up to 3 weeks or longer, or whether there is a constant fresh cyclical formation of these structures. Certainly there appears to be some influence at work sufficiently powerful to interfere with the natural development of the agamonts into gamonts but more particularly the gamonts into gametocytes. We agree with Cowdry & Danks (1933) that further study on agamonts and gamonts is urgently needed. The statement of these workers that "we have no data at all bearing on factors which facilitate or retard the

change of agamonts into gamonts" is amply supported in our investigations. The length of life of an agamont or a gamont is quite unknown.

The interesting and important question in turning sickness, however, is why do not the gamonts develop into gametocytes, the forms that parasitise the red blood corpuscles? Why does the parasite not follow the usual life history of *Theileria* in its mammalian host? There seems to be obviously some agent sufficiently strong to arrest or greatly impede further development of the gamonts. That it is not merely a question of time is obvious enough when one remembers that most cases of turning sickness live up to a week or longer, while Cowdry has shown that in the case of *T. parva* the whole cycle from immature agamont to the appearance of mature blood forms may take place on an average in 1·6 days.

Since there is plenty of evidence that turning sickness occurs in animals that have had East Coast fever and are therefore immune, one might consider that there is in the blood some immune body which has definite gametocidal properties. Unfortunately there is no experimental proof of any such substance. Not only has it been impossible to demonstrate any immune bodies in the serum of cattle recovered from East Coast fever but the inoculation of massive doses of serum of recently recovered animals into early cases of the disease showing schizonts has no effect of any kind.

Lichtenheld, quoted by Schilling and Meyer (1930), demonstrated by the complement fixation test that no antibodies were present in the serum of animals recovered from East Coast fever.

Not the least remarkable phenomenon of turning sickness is the great scarcity of schizonts in the spleen and lymphatic system. Except in Case IV, it required long and careful search to find Koch's bodies in these organs and in most cases they could not be demonstrated at all. This is an interesting feature, since in all the theileriases the schizogonous stages of the parasites occur in the lymphatic system, particularly, as Cowdry & Danks (1933) and Steck (1928) have recently emphasised, in the medium-sized and large lymphocytes. Meyer (1911), and the earlier workers, believed that the endothelial cells were also parasitised, but this view is now discredited, though it is still repeated in many modern text-books.

As regards the blood it was unusual to find *Theileria* in any numbers in the red cells. Indeed, these parasites were generally extremely rare and it was impossible to say whether those present were of the *mutans* type, or gametocytes formed from the schizonts in the brain. In two Cases (IV and XVIII) they were present in increased numbers. While there is some doubt about Case IV being turning sickness, there is none about Case XVIII whose East Coast fever history is known. As will be seen from the records this animal contracted turning sickness exactly 3 months after recovery from East Coast fever. The heavy blood infection cannot, therefore, be connected with East Coast fever, since *Theileria parva* does not persist in the blood after recovery. There is no doubt that this particular animal was exceptional in

presenting such a high degree of parasitisation of the erythrocytes, though there is no evidence that the gametocytes would have been capable of further development in suitable ticks. It is unfortunate that no ticks were collected off this case for transmission purposes.

The morphology of the *Theileria* in this animal was carefully studied and a comparison made with the forms of *T. parva* observed by Cowdry & Danks (1933) in the red blood corpuscles of cases of East Coast fever in Kenya.

According to these investigators "plump" forms of the parasite predominate in the red blood cells in East Coast fever. In twelve animals the blood parasites were very carefully examined by Cowdry, and in eight of them the number of "plumps" exceeded the "tailed" forms. In the other four the "tailed" forms were in excess. It is quite unknown whether the prevalence of any particular form possesses any special significance. In our investigation calf 601 (Case XVIII) had a count as follows:

"Tailed" forms 102; "plump" forms 26; "spherical-ovoid" parasites 17; "rod-shaped" forms 5; and "dumb-bell-shaped" forms 1. In this animal the agamonts totalled 66·4 per cent. of all the schizonts. Such a great difference in number between the "tailed" and "plump forms" of this animal has no analogy with any of Cowdry's twelve East Coast fever cases. It may be stated, however, that in the East Coast fever investigations carried out at Entebbe it was not rare to find very great differences between the number of "plump" and "tailed" parasites. In some cases the "tails" were more numerous and in others the "plumps" were in excess. Further observations are being made on this subject.

In three animals (Cases IV, VIII and XIX), schizonts were found in large numbers in the peripheral blood mostly in the agamogonous state in the larger lymphocytes. In these animals *Theileria* was not common. Anaemic changes were not observed in the blood, but no differential counts were carried out.

## IX. PATHOLOGICAL HISTORY

The predominant lesion in the brain and pia mater is a plugging or blocking, partial or complete, of the smaller arteries and arterioles by dense masses of cells of the lymphocytic series. In some cases red cells may be intermingled with the lymphocytes, but it is common to find the lymphocytes alone forming veritable emboli. Under high magnification, bodies indistinguishable from the schizonts of East Coast fever can be easily made out in the cytoplasm of the lymphocytes. These schizonts vary in size and are most numerous in very acute cases. In animals that have shown symptoms for some time they are more difficult to find, and in chronic cases none are seen at all. They disappear if the brain shows the slightest signs of commencing decomposition. Both agamogonous and gamogonous forms can be made out. By far the greatest number are intracellular within medium-sized lymphocytes, but it is not rare to find a few lying free within the vessels. They have never been seen to parasitise the vascular endothelium. Indeed, except for

mechanical changes caused by greatly increased intravascular pressure, the endothelial cells show no change. It is now accepted that in East Coast fever and other theileriases, schizogony of the parasite is confined to the cells of the lymphocytic series. Mitotic changes are not rarely observed in the lymphocytes within the blocked capillaries.

The size of the emboli or lymphocytic plugs vary within wide limits, from a collection of a few schizonts bearing lymphocytes in the capillary system surrounding the nerve cells, to a large embolus in a medium sized artery often visible to the naked eye.

The emboli are responsible for extensive blocking of the blood vessels in the brain over a large area and give rise to serious vascular changes of which thrombosis is very apparent. In some cases this is very widespread and vessels may be overloaded to a remarkable extent with partially or completely thrombosed blood. If the vessel is hopelessly obstructed and the circulation completely arrested the thrombus soon shows signs of degeneration.

A large number of red blood corpuscles may be seen in greatly dilated capillary spaces in the brain tissue itself, but it is uncommon to find leakages of blood from the vessels, no matter how engorged they may be. The red petechiae seen on naked eye examination of the brain are greatly dilated vessels containing whole blood, or thrombi, or, in not a few cases, thrombi formed of red cells side by side with plugs of parasitised lymphocytes.

Undoubtedly some of the over-distended vessels finally give way, causing large haemorrhages which overflow and swamp extensive areas of brain tissue. Such extravasations occurred in Cases I and XVI. It is not known exactly what vessels were ruptured and responsible for these massive lesions, but it is significant that the haemorrhages occupied the same situation as those which follow rupture of the lenticulo-striate artery in the human subject.

Another, but by no means constant lesion, is the appearance in the adventitial tissue of the vessels of an increased number of cells which give the picture of perivascular "cuffing". This may be quite marked, and in our opinion it is the results of proliferation of lymphocytes in the lymphoid spaces of the adventitia.

In our sections these "cuffs" only exceptionally contained schizonts, and this seems to show that they are not derived from the lymphocytes within the vessel itself but have an extravascular origin. Moreover, all the cells are not medium-sized lymphocytes. A large number of plasma cells are present, while glial cells are not rare. The origin of these "cuffs" requires further investigation. They are rarely noticed about the larger vessels in the pia mater, and seemed to be confined to the brain.

In the neighbourhood of the large haemorrhages in Cases I and XVI, blood, often in considerable quantities, could be demonstrated in the lymph spaces of the adventitia. It is well known that when a large haemorrhage occurs in the brain many small vessels are torn, with the result that blood passes into the lymph channels of the vessels. Further, blood may also be actually drawn

back for a considerable distance along the lymph sheaths from the main haemorrhages themselves. The second appears to be the correct explanation in turning sickness for two reasons, viz. (*a*) adventitial blood is most abundant near large haemorrhages, and (*b*) the blood is always within lymph sheaths and not scattered diffusely over the adjacent tissues.

As regards the brain tissue itself, the neurons near the large haemorrhages or in the immediate vicinity of the greatly distended vessels show degeneration due to increased pressure. They are swollen, very granular, and considerable chromatolysis is present. Often very distorted, they eventually fragment and finally disappear completely.

With the subsidence of the acute symptoms reparative changes occur, but there is never any replacement of nervous tissue. Cavity or cyst formation is a constant feature, their walls being formed by proliferation of neuroglial tissue. All traces of haemorrhages, lymphocytic, embolic and thrombosed vessels are to a great extent removed, while the neurons show very serious degenerative changes or may not be seen at all.

The longevity of these chronic cases depends on the amount and site of the brain tissue destroyed. Thus blindness, which is so prominent a feature of these animals, is associated with cavity formation and wholesale destruction of nervous matter in the visual tracts. The most constant pathological lesions of chronic cases are atrophy, cavity formation and proliferation of neuroglia which shows a tendency to become sclerotic.

Microscopical examination of the spleen, lymphatic system and the various parenchymatous organs showed nothing beyond a slight, but not constant, degree of stasis. Prolonged search failed in all cases to reveal the presence of schizonts in these structures.

### *Discussion*

The plugging of cerebral capillaries by emboli of protozoan parasites has not been hitherto reported in animal pathology, though an analogy exists in human medicine in the cerebral form of malaria in which enormous numbers of sporulating forms of *Plasmodium falciparum* are found in the capillaries of the brain, producing grave nervous symptoms and pronounced pathological lesions. Judging from the available literature, however, the macroscopic cerebral lesions are never as extensive in cerebral malaria as they are in turning sickness.

Zoologically, the parasites causing these diseases are comparatively closely related. They both belong to suborders of the Coccidiida. They agree in reproducing in the mammalian host by schizogony which occurs, in the case of *Plasmodium falciparum*, in the red blood corpuscles and, in turning sickness, as in all the theileriases, in cells of the lymphocytic series. While the nature of the cerebral schizonts in pernicious malaria is known with certainty to be the sporulating stage of *P. falciparum*, considerable doubt still exists about the ultimate life history of the schizonts in turning sickness. It is quite

unknown for what length of time they can persist in this phase, or whether, after a time, they retrogress, or, again, whether they give rise to mature gametocytes which invade the blood stream and are subsequently ingested by ticks in which they continue sporogonous development. These are questions that require further investigation.

The origin of these schizonts in turning sickness is shrouded in mystery. It is clear that they must have developed from sporozoites inoculated at some period by infected ticks, but there is no evidence to show whether they are the result of recent infection or whether they have lain dormant since a previous attack of East Coast fever. The whole subject is a most perplexing one and lends itself to interminable theoretical speculation.

As pointed out previously in this paper the brain is only slightly affected in East Coast fever or the other theileriases, and it is rare to find schizonts at all in smears or sections of the organ. This makes it all the more astonishing to find large numbers of schizonts and such grave lesions in this organ in turning sickness.

## X. Transmission experiments

Up to the present every attempt to transmit the disease artificially has failed. The following experiments have been conducted, viz.:

### A. *With brain tissue*

Whenever a living case of turning sickness was available two series of experiments were initiated: (*a*) in which healthy adult cattle, with a definite history of recent recovery from East Coast fever, were used, and (*b*) calves, which had been born and maintained since birth under tick free conditions.

The turning sickness case was destroyed by pithing and its brain rapidly removed and placed in sterile Ringer solution at 38° C. The brain was rapidly emulsified, strained through sterile butter muslin, and without loss of time inoculated into experimental calves which, prepared in advance, were ready at hand. Every possible inoculation route or combinations of routes were used, namely, subdurally, intracerebrally, intradermally, intraglandularly, intrasplenically, intravenously and subcutaneously. The need for speed in the preparation of the brain emulsions and in carrying out the inoculations was recognised, because we were dealing with a very delicate protozoon, whose viability and longevity outside the body is quite unknown. Nevertheless, our transmission experiments were all negative. It may be mentioned that K. F. Meyer (1909) was the first to transmit East Coast fever by implanting spleen or a piece of spleen from an infected animal into the peritoneal cavity of a susceptible beast. As this work has been repeatedly confirmed it is evident that the schizonts survive rapid transition from the body of one animal to another, and our negative results with the brain of turning sickness animals cannot be entirely explained on the assumption that the schizonts were destroyed by external influences.

### B. *With blood*

In two cases of turning sickness Koch's bodies were found in the lymphocytes of the peripheral blood, and on several occasions attempts were made to see whether the disease could be set up in animals both susceptible and recovered from East Coast fever by massive intravenous doses of citrated jugular blood.

On no occasion were any positive results obtained. Similarly, blood given intraperitoneally and intracutaneously was equally unproductive of results. The difficulty, of course, in these experiments is to be certain that the blood of the cases of turning sickness actually contained schizonts, which might be so rare as to escape ordinary microscopic examination of stained films.

### C. *With ticks*

The tick population was very much the same on all the animals. The common brown tick (*Rhipicephalus appendiculatus*) in larval, nymphal and adult stages, predominated. Less numerous were the blue tick (*Boophilus decoloratus*), and the variegated tick (*Amblyomma variegatum*). Occasionally *Rhipicephalus evertsi* and *Amblyomma cohaerens* were recognised but never in great numbers.

During the present investigation attention was concentrated on *Rhipicephalus appendiculatus*, which is by far the commonest vector of East Coast fever in this part of Africa.

Several experiments showed that it was impossible to transmit turning sickness by feeding this tick on calves recovered from East Coast fever. In addition it would appear that such ticks were also free from the virus of East Coast fever, since they failed to infect clean calves which, subsequently, proved susceptible on exposure to natural infection.

Tick emulsion made from ticks collected on sick animals at all phases of their life history also failed to transmit.

## XI. GENERAL DISCUSSION

Cerebral symptoms and lesions have not been described as occurring in East Coast fever, though apparently both have been recorded in other forms of theileriasis. As far back as 1904 Dschunkowsky & Luhs, in their first publication on tropical theileriasis, mention that severe brain disturbances as shown by clinical nervous symptoms frequently occur. It is now claimed that these investigators were working with a mixed infection, but their observation is of considerable interest.

Velu (1921) described the prevalent form of theileriasis in Morocco, and in the acute form of the disease records that it was not rare to observe cerebral symptoms. Later, French workers in Algiers investigating *Theileria dispar* infection also noted the occurrence of severe clinical cerebral symptoms such

as mania, circular movements, etc., during the later stages of the disease. None of these workers describe in detail the pathology of the brains of their cases.

Nevertheless, it is of very great interest that nervous symptoms should be of such frequency in the Mediterranean theileriases, since they are not observed in classical East Coast fever as observed in South East and Equatorial Africa.

It is not intended to discuss at any length in this paper the vexed and controversial question of the validity of the various species of *Theileria*. That is a problem for systematists and has recently been dealt with by du Toit (1930), but the occurrence in Central Africa of a well-defined protozoan disease characterised by nervous symptoms raises the question whether the Mediterranean species of *Theileria* does not extend further than is at present accepted.

Careful observation over a period of 4 years shows that in Uganda, at any rate, two well-defined species of *Theileria* exist, the innocuous *T. mutans* and the pathogenic *T. parva*, and that there is no evidence of the existence of *T. annulata* or *T. dispar*. In nearly every point the Uganda theileriasis differs from *T. dispar* or *T. annulata* infections, and we can confidently conclude that turning sickness in this country is not associated with the Mediterranean or Asian forms of theileriases.

It must, however, be repeated that the connection or relationship of East Coast fever and turning sickness is still problematical and will only be decided after further extensive experimentation.

Reference to our case records will show that of the twenty-four animals affected with turning symptoms no less than fifteen (approx. 63 per cent.) were known to have had this disease comparatively recently. More careful analysis of the histories of these fifteen cases shows that nine had had East Coast fever 6 months previously, three within 9 months, two within a year, and one as long back as 19 months. Of the remaining nine, three were aged beasts with a history of East Coast fever some years before, while in six it was impossible to obtain any kind of history though all came from known enzootic districts.

It seems necessary at this stage to examine at length the possibility of turning sickness being either (1) a chronic protracted form of East Coast fever, or (2) a relapsed or latent form of the disease. It has never been asserted in this paper, or at any other time, that we have had to deal with a new species of *Theileria*, but we have kept in mind the contention of du Toit (1930) that there may be wide variations in virulence within a species.

It will be remembered that early in the present paper a brief account was given of the epizootiology of East Coast fever as it occurs in Uganda, and it was pointed out that some 20 per cent. of all cattle suffer from the disease in a very mild clinical form. These cases have some analogy with the experience of Velu (1921, 1933) in Morocco, and Brumpt in Tunis. In Brumpt's strain

the infection, was, at first, always mild and chronic and then, after a considerable lapse of time (about 3–7 months) suddenly became peracute and fatal in a certain percentage of cases (du Toit, 1930).

We have no experimental evidence to show that the mild cases met with in Uganda run a chronic course, but it is of interest to note that the majority of our cases developed turning sickness within 2½–9 months of their initial East Coast fever infection, and that this period closely synchronises with the time given by Brumpt in his cases, which developed acute symptoms after a preliminary chronic course.

All our mild cases remained in a heavily enzootic area for the rest of their life and were constantly exposed to infected ticks, so that the possibility of a massive infection on top of a preliminary mild attack must not be overlooked.

It is quite possible that some of the mild cases do run a chronic inapparent course which finally terminates in an acute attack of turning sickness. This is conjecture, since at present we have no experimental or clinical evidence of such a form of East Coast fever in this country, and our views in this respect are supported by those who maintain that a chronic form of East Coast fever does not exist (du Toit and others).

Nevertheless, it must be pointed out that van Saceghem (1924) has described a chronic form of the disease in the mandated territories of Ruanda-Urundi and the Kivu area of the Congo which lie to the south of Uganda. In this very same country Lichtenheld, before the world war, made precisely similar observations. There is thus evidence that a chronic form of East Coast fever does actually exist in parts of equatorial Africa. The prevalent opinion as expressed in the literature that "amakebe" is a mild disease should no longer be accepted. Van Saceghem (1924) states: "Bruce et ses collaborateurs la trouvent en l'Ouganda, une forme relativement benigne." Such statements have found their way into text-books and are repeated in every new edition. It is time that the real nature of "amakebe" was more fully appreciated.

East Coast fever is the most serious cattle disease in this country and is responsible for a higher annual mortality than either rinderpest or trypanosomiasis. Even in highly enzootic areas the mortality may exceed 40 per cent., but where the sick calves are neglected the figures are much higher. In 1922 an extension of this so-called "mild disease" spread into a clean district and was responsible for a holocaust which has not been exceeded in severity in any other part of Africa. What appertains in Uganda at present is that the indigenous cattle have acquired some resistance as the result of centuries of exposure and *not that* the parasite has fallen off in virulence. Repeated attempts to keep grade or pure-bred cattle in "amakebe" areas have proved disastrous on every occasion that the experiment has been tried. It can be accepted without hesitation that *T. parva* in Uganda is still a very pathogenic organism, and mild cases of East Coast fever, if such occur at all, are exceptional.

There is no evidence to support the view that turning sickness may re-

present a relapse or breakdown to East Coast fever. No relapses have ever been seen at this Laboratory, though some of the district veterinary officers report that they do rarely occur.

One must be careful to differentiate between relapses and reinfection. While many deny the existence of the former they believe in the latter, and such an eminent authority as Theiler "has on several occasions observed that recovered animals succumb to a renewed exposure on tick infected premises; second and possibly third attacks may occur but they are rare" (Meyer, 1930). The point is naturally one of very great importance for the veterinary control of the disease.

Within recent years Bevan (1924) in Southern Rhodesia and Purvis (1927) in Kenya have questioned the permanence of a sterile immunity in East Coast fever. The former investigator has propounded a theory of latency in which he considers that the parasites of East Coast fever may lie dormant in the tissues of recovered animals, perhaps, in some inapparent form, awaiting their chance for further proliferation when the influence or factor, which inhibits their continual activity, is removed. Bevan has produced no experimental evidence in support of his views, which are not acceptable to most authorities (Cowdry & Danks, 1933; Meyer & Schilling, 1930; and others) because he has not eliminated all possibilities of reinfection. However, Bevan's theory has led to intensified research into the immunity of East Coast fever, particularly in South Africa where du Toit (1931) carried out drastic experiments in his efforts to produce genuine relapses or breakdowns. His results served to confirm what has been accepted for many years, namely, that the immunity following an attack of East Coast fever is solid, permanent and sterile. At Entebbe certain investigations (as yet unpublished) support du Toit's conclusions. We have subjected East Coast fever immune calves to the ordeal of acute rinderpest. We are convinced that no severer provocative could be found, since rinderpest virus not only produces the gravest and most widespread lesions throughout the body but the virus itself has a predilection for the white blood corpuscles, in which it is actually intracellular (Todd, 1930). In this position the virus is likely to come into contact with any dormant or latent schizogonous forms of *T. parva* which one might suppose to lie up in the lymphocytes, since it is in these cells that active schizogony of the parasite occurs during the acute paroxysm of the disease. Our experiments remained negative in so far as producing relapses or breakdowns were concerned, and it would at least seem as if latent infections must be extremely rare, though it is more probable they do not occur at all. During the last 4 years innumerable cattle used in the making of rinderpest virus, all immune to East Coast fever, have been kept under close observation at this Laboratory, and not one has relapsed to East Coast fever.

The whole question of reinfection requires to be thoroughly reinvestigated, particularly to test the view, widely held in Uganda, that cattle moved from one enzootic area to another relapse to East Coast fever.

It is also necessary to know if different strains of *T. parva* exist which produce a solid group immunity but an unstable specific immunity. These are questions that are repeatedly asked in this part of Africa and to which at present no definite reply can be returned, especially in view of du Toit's recent pronouncement that there are wide variations in virulence within a species. In our opinion the solution of the nature of turning sickness may be elucidated during further investigations into this aspect of East Coast fever.

## XII. SUMMARY

1. A specific disease of cattle is described which occurs sporadically in the East Coast fever areas of eastern equatorial Africa. Clinically, acute cases are characterised by peculiar nervous symptoms, absence of fever and blindness. Death occurs in most cases within 2 to 21 days of the onset of illness. Chronic cases are encountered from time to time and may survive up to 6 months.

2. The disease is widespread in Uganda. It affects cattle of both sexes and all breeds, and is commonest in young stock between 6 months and 2 years of age. It has, however, been observed in older animals.

3. Pathologically, in acute cases petechial haemorrhages or extravasations are found in various parts of the brain and meninges. Generally the haemorrhages are small and do not seriously injure the adjacent brain tissue. In some cases the extravasations are responsible for a considerable amount of damage.

4. Microscopic examination shows that the brain lesions are due to extensive embolism of cerebral vessels. The lymphocytes which form these emboli contain in their cytoplasm protozoan bodies indistinguishable in structure from the schizonts of the genus *Theileria*. These bodies occur in great numbers in the brain, but are extremely rare or absent in the lymphocytes of other parts of the body.

5. In chronic cases the haemorrhages have been absorbed but the brain is permanently damaged by replacement changes, and by the appearance of small cysts or cavities which contain a citron-coloured fluid. These cavities when numerous seriously disorganise certain parts of the brain.

6. The disease has not yet been transmitted experimentally, and its connection with the theileriases is still uncertain. However, much evidence has been collected to show that cases of turning sickness have been infected with East Coast fever some time previously, generally on an average of 6–8 months.

7. The possible relationship between turning sickness and East Coast fever is discussed at some length during the course of the paper.

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Fig. 1.

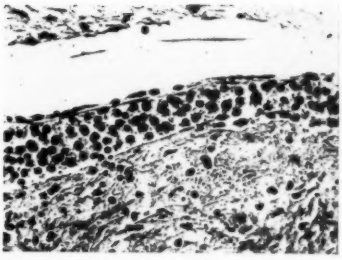


Fig. 3.

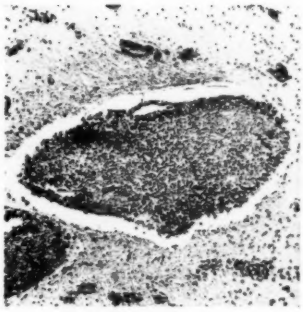
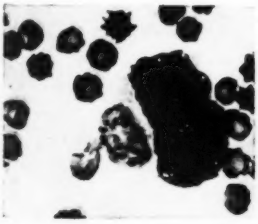


Fig. 4.



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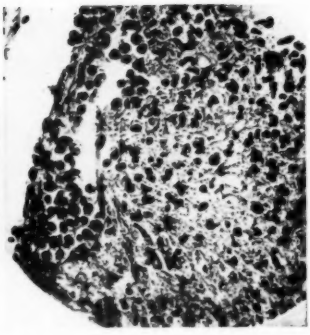

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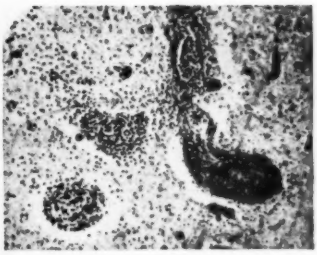

Fig. 6.

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## EXPLANATION OF PLATE XII

(Photographs)

Fig. 1. Brain of Case I; mounted museum specimen showing large haemorrhage in occipital lobe above lateral ventricle. A second smaller haemorrhage is seen in the white matter of the anterior lobe. Natural size.

Fig. 2. Smear from cerebral vessel of Case XIV, showing intracellular agamont. ×1100.

Fig. 3. Small cerebral capillary showing lymphocytic embolism. Practically every cell contains a schizont. ×240.

Fig. 4. Cerebral cortex with thrombosed vessels. The thrombus shows commencing organisation while along its periphery are arranged masses of schizont-bearing lymphocytes. A few of these cells can be seen in different parts of the thrombus. ×60.

Fig. 5. Thrombus in previous section; higher magnification of top corner. To the left are schizonts bearing lymphocytes. ×240.

Fig. 6. Cerebral cortex showing a group of small capillaries. Two of the larger vessels give the appearance of perivascular cuffing. It is exceptional to find schizonts in the lymphocytes of these cuffs even when they are numerous in the intravascular lymphocytes. Some of the smaller vessels are plugged entirely by red blood corpuscles and others by lymphocytes. ×60.

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# SYMBIOTIC BACTERIA IN A BLOOD-SUCKING INSECT, *RHODNIUS PROLIXUS* STÅL. (HEMIPTERA, TRIATOMIDAE)

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*From the London School of Hygiene and Tropical Medicine*

(With 4 Figures in the Text)

It is well known that many blood-sucking insects regularly contain intracellular micro-organisms (Buchner, 1930). The constant occurrence of these, and the mutual adaptations between them and their host, has led to the belief that they are symbionts which perform some necessary function in the insect's economy. It was at first suggested that they were concerned with the digestion of blood. But in the case of the tsetse fly (*Glossina*), which was held to give the main support for this idea (Roubaud, 1919), it has been shown that there is no digestion and no digestion enzymes in that segment of the intestine where the symbionts lie (Wigglesworth, 1929). It was pointed out independently by Wigglesworth (1929) and Aschner (1931) that symbionts occur only in those blood-sucking insects which feed solely on blood at *all* stages of their life cycle; these authors therefore suggested that the micro-organisms might "furnish their host with some accessory food substance comparable or possibly even identical with the vitamins necessary for mammalian growth" (Wigglesworth, 1929).

In recent years evidence for this hypothesis has been accumulating. (i) It has been shown that sterile blood is an insufficient diet for mosquito larvae, but it becomes sufficient if infected with bacteria (Aschner, 1931; Rozeboom, 1935). (ii) It was shown by Aschner (1932) that if the louse *Pediculus* is deprived of its symbionts by operative removal of the "mycetome" which contains them, its powers of nutrition and reproduction are greatly impaired, and that this impairment may be made good, to some extent at least, by the addition of yeast extract to the diet (Aschner & Ries, 1933; Ries, 1933). (iii) Hobson (1933) proved that sterile blood is an inadequate diet for blow-fly larvae (*Lucilia*), but it can be made adequate by the addition of "vitamin B."

There is one group of exclusively blood-sucking insects in which symbiotic organisms are said to be absent (Buchner, 1923). These are the Triatomidae, containing the genera *Rhodnius* and *Triatoma* and their allies. The object of the present paper is to record the existence of micro-organisms in this group and to describe some preliminary experiments which support the view that they are a source of vitamin.

### DEVELOPMENTAL CYCLE OF THE BACTERIA

Duncan (1926), when studying the gut contents of a few specimens of *Rhodnius prolixus*, found that they all yielded a Gram-positive bacillus. This organism has been found to occur in every individual examined, to be transmitted through the egg, and to have an intracellular and an extracellular cycle.

Fig. 1 shows the gut of *Rhodnius*. There is a short oesophagus; a mid-gut consisting of three portions: a narrow segment (*mg.* i), a greatly dilated "stomach" (*mg.* ii) with a posterior sphincter and a long coiled "intestine"

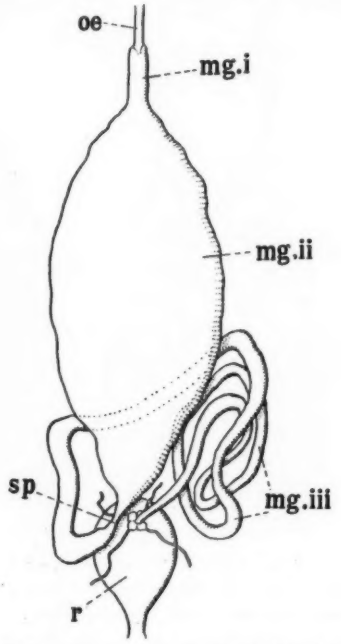


Fig. 1. Alimentary canal of *Rhodnius*. *mg.* i. segment of mid-gut containing intracellular bacteria; *mg.* ii. "stomach" (mid-gut); *mg.* iii. "intestine" (mid-gut); *oe.* oesophagus (fore-gut); *r.* rectum (hind-gut); *sp.* sphincter.

(*mg.* iii), which becomes gradually narrower before it enters the pyriform rectum that forms the hind-gut. The large meals of blood are stored in the stomach without undergoing digestion: the corpuscles may remain intact for several days and the haemoglobin remains red for several weeks. Digestion takes place in the intestine, the contents of which are always dark brown or black.

In the newly hatched *Rhodnius* there are no micro-organisms in the lumen of the gut; but the cells lining the anterior narrow segment of the mid-gut (*mg.* i) are stuffed with intracellular bacteria (Figs. 2 A and 4 A). After feeding,

these cells become still more swollen, and the micro-organisms probably multiply; but it is usually not until after the insect has moulted, that is, about 10 days after its meal[1], that they are set free into the lumen. This process is shown in Figs. 2B and 4B. Vesicular swellings filled with micro-organisms are extruded, often through the striated border of the cells, and disintegrate in the gut cavity. Soon the lumen of the narrow part of the mid-gut is filled with masses of deeply staining bacteria which extend into crypts between the cells (Figs. 2C and 4C) and invade the blood in the stomach.

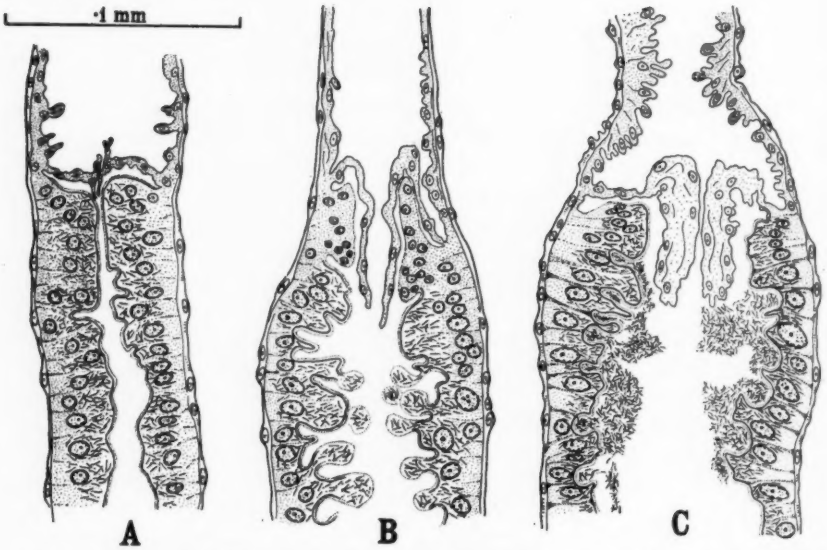


Fig. 2. Longitudinal sections of the junction between fore-gut and mid-gut. A, first-stage nymph, recently hatched, unfed; B, first-stage nymph, 7 days after feeding; C, second-stage nymph, 10 days after moulting (about 3 weeks after feeding).

When *Rhodnius* again sucks blood, clumps of these organisms are often washed into the stomach. In sections of insects fixed 10 min. after feeding, the leucocytes in the ingested blood are found to have massed themselves round these clumps, and many of the organisms are being taken up by the polymorphonuclear cells (Figs. 3A and 4D). It was, indeed, these masses of leucocytes which first directed attention to the bacteria. Three or four hours later the leucocytes and bacteria have become scattered again by the peristaltic movements of the stomach, and the bacteria are difficult to recognise in sections. After 3 or 4 days (at 24° C.) numerous minute colonies are visible in the sections (Fig. 3B). These gradually increase in size, and by 14 days after feeding some of them are very large and dense (Fig. 3C). At the end

[1] *Rhodnius* requires only a single meal in each instar.

of a month there is little blood in the stomach, but what remains is full of colonies. The organisms are visible also in the blackened blood in the intestine. They could not be recognised in the rectum nor in smears from the faeces; they must therefore be entirely digested.

The organisms persist inside and outside the cells throughout the life of *Rhodnius*, and they are transmitted through the egg; but the details of this transmission have not been worked out.

Similar organisms occur in *Triatoma rubrofasciata* de Geer, *T. infestans* Klug. and *Eutriatoma flavida* Neiva.

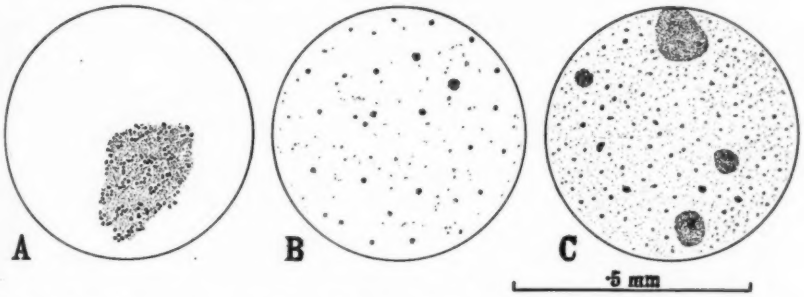


Fig. 3. Sections of the blood in stomach of adult *Rhodnius*. A, 10 min. after feeding, showing mass of bacteria invaded by leucocytes; B, 8 days after feeding, showing small colonies of bacteria; C, 14 days after feeding, showing some large colonies.

### MORPHOLOGY AND CULTURE OF THE BACTERIA

The organism has the characters of a diphtheroid bacillus. In the intracellular stage it is in the form of little banded rods which do not stain very conspicuously. On becoming extracellular they stain more deeply, and resemble typical diphtheroids in their variable forms and banded staining. As the colonies in the stomach become larger, *e.g.* at 3 weeks after feeding, the bacteria become so conspicuously beaded as to resemble strings of cocci (Fig. 4D), and involution forms of many kinds are present (Fig. 4F). Finally, these strings break up, and the coccoid bodies are no longer strung together but lie in clusters. This state is most common in the intestine where digestion is in progress, and it is uncertain whether these coccoid bodies are still viable.

When cultured on glucose-agar the organism grows rather slowly at 30° C., but it goes through similar changes. In young cultures they are typical diphtheroids; in old cultures long thread-like forms and conspicuously beaded forms predominate. When stained by the Ziehl-Neelson method they are non-acid fast to 1 per cent. $H_2SO_4$; they are weakly Gram-positive, appearing rather pale after short decolorisation with alcohol.

As described above, there is no doubt that the bacteria are transmitted through the egg. But a few attempts to culture them from crushed eggs failed. It is possible, therefore, that the intracellular form cannot be cultured.

### The function of the bacteria[1]

Hobson (1933) has shown that sterile serum is a deficient diet for *Lucilia* larvae unless it is supplemented by the addition of yeast extract or an autoclaved extract plus a vitamin $B_1$ concentrate. Dr Hobson has carried out some preliminary experiments to see whether this deficiency could be made good if the serum was first contaminated by the bacteria from *Rhodnius*.

A specimen of sterile horse serum was divided into two parts, one kept sterile and the other infected with the bacterium from *Rhodnius* and incubated at 23° C. for 48 hours. Smears prepared at the end of this time showed many colonies of the rod-shaped forms with banded staining (Fig. 4E).

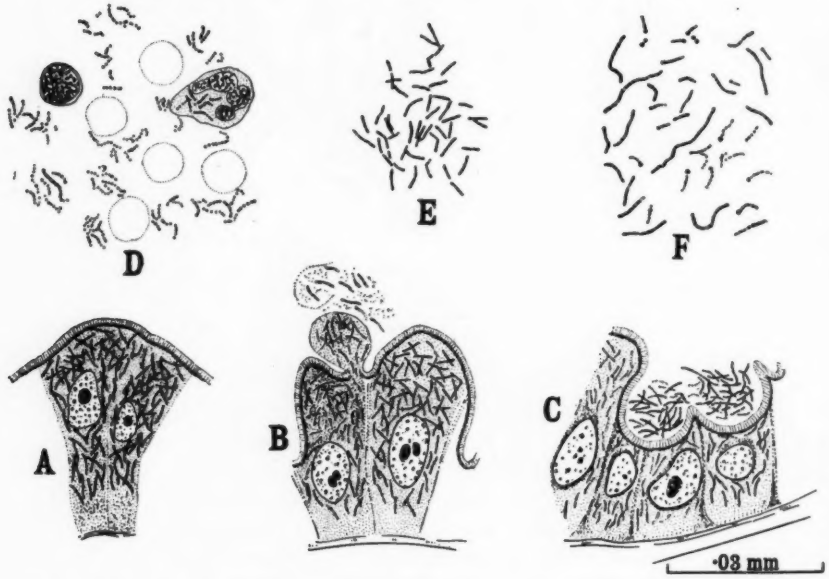


Fig. 4. A, B and C are from the same preparations as Fig. 2 A, B and C. They show the intracellular bacteria becoming extracellular. D, smear from stomach contents 45 min. after feeding, showing beaded form of bacteria being ingested by a polymorphonuclear leucocyte; E, 48-hour culture of the bacterium in serum; F, smear from stomach contents of second-stage nymph 3 weeks after first feed.

Nine culture tubes for *Lucilia* larvae were prepared by adding 0·3 c.c. of serum to a little ball of cotton-wool in each, and introducing two sterilised *Lucilia* eggs. Sterile serum was used in three tubes, serum infected with the organism from *Rhodnius* in three tubes, and sterile serum plus yeast extract[2]

[1] These experiments were carried out by Dr R. P. Hobson.

[2] An extract of non-autolysed yeast was used. Still more rapid growth is obtained if autolysed yeast is employed.

in three tubes. At the end of 6 days the larvae in the sterile serum had grown to about four times their original weight; those in the infected serum to about forty times; those receiving yeast extract to about 120 times. The larvae developing in the same mixtures behaved alike in each case.

Although these results ought to be repeated on a larger scale, they strongly support the view that the bacteria in *Rhodnius* provide the "vitamin B" which is lacking in blood.


## SUMMARY

A bacterial organism is described which occurs constantly in *Rhodnius prolixus*. It is present in the unfed newly hatched insect inside the cells at the cardiac end of the mid-gut. Some days after feeding, the bacteria are set free into the cavity of the gut and multiply in the undigested blood in the stomach. They are ultimately digested in the intestine.

Morphologically they resemble diphtheroid bacilli in being highly pleomorphic and in giving rise to thread-like forms in old cultures.

Preliminary experiments with *Lucilia* larvae by Dr R. P. Hobson, suggest that when blood is infected by this micro-organism it contains "vitamin B" and becomes an adequate diet for insect growth.

These observations support the view that symbiotic organisms in exclusively blood-sucking insects provide an endogenous source of vitamin.


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# A NOTE ON THE ADULT FEMALE OF *ANGUILLULINA APTINI* (SHARGA), A NEMATODE PARASITISING *APTINOTHRIPS RUFUS* GMELIN

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(With 1 Figure in the Text)

*Anguillulina aptini* (Sharga) was described in 1932. Nematodes had previously been recorded in other species of thrips (Uzel, 1895; Russell, 1912), but no descriptions had been published. There is an extensive body of literature dealing with the nematode parasites of other insects (see van Zwaluwenburg, 1928).

About 17,000 specimens of *Aptinothrips rufus* Gmelin were dissected by the writer in the course of an investigation into the factors governing the distribution of the nematode; and after examining a great number of the female parasites it seemed clear that they differed in some respects from those described by Sharga. I am satisfied that I am working on the same species as that described by him, and on first sight the reproductive system is as in his drawings, but when care was taken to avoid all crushing an additional coil in the ovarian tube was seen in all the mature specimens examined (Fig. 1).

The young female when ready to leave the host is elongate and cylindrical, with a bluntly rounded head and a slightly tapering tail. It measures about 0·35 by 0·018 mm. It can be distinguished from the larval male, as the latter possesses caudal alae when it is this size and moves in a peculiar jerky way. The young female moves much more slowly, and in a somewhat sinuous fashion.

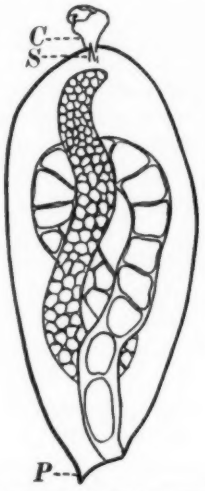
If slight pressure be applied to the abdomen of an infected thrips young males and females are often extruded from the anus. I think therefore that fertilisation takes place in the free-living stage. In numerous cases insects have been found to contain a single adult female.

Both larval and pupal thrips have been found to be infected. The method of attack is probably similar to that described by Bovien (1932) for *Scatonema wülkeri* Bov. in *Scatopse fuscipes* Meig., that is that the nematode penetrates the body wall of the insect by means of the stylet.

A post-nuptial moult occurs when the female has entered the body cavity of the host. The old skin is seldom discarded altogether and usually remains attached anteriorly in a state of greater or lesser dilapidation. At this stage the stylet is conspicuous, and the rest of the alimentary canal, somewhat diminished in diameter, is often clearly seen. This rapidly degenerates till all

that remains is the stylet, a shrunken anterior part of the oesophagus leading into a small bulb-like structure, and a formless mass of cells more or less central in position. At the posterior end of the animal lies an oval body, the gonad. As the cells of the alimentary canal degenerate and are absorbed the gonad becomes greatly elongated and finally appears as a tube-like structure folded upon itself.

While these changes are taking place the general aspect of the nematode has altered considerably. When the final moult is complete a certain amount of swelling occurs and a simultaneous decrease in length, so that the cuticle is thrown into folds giving the animal an irregularly annulated appearance. Sinuous movements show that some part of the muscular system is still




Text-fig. 1. *Anguillulina aptini*, female (0·223 × 0·119 mm.) showing reproductive system, slightly diagrammatic. *C*, remnant of cubicle from last moult; *S*, stylet; *P*, tail papilla.

functioning. Finally considerable swelling takes place, the corrugations of the cuticle are filled out, and the mature female appears as a distended sac-like organism (Fig. 1) almost incapable of movement. Posteriorly the tail persists as a small pointed papilla. Anteriorly the cuticle shed at the last moult is often visible, sometimes in the reduced condition figured, sometimes as an appendage as long as, or longer than, the rest of the animal. At the base of the sheath the stylet remains, but there is no other trace of the alimentary system.

In a specimen viewed from the right side, the ovary extends back from the anterior end of the organism in a simple blunt **S** shape. The tail of the **S** twists to the right and under itself and passes forward as if to form a figure of eight. The ovary then passes into the oviduct which runs in a slight curve to the uterus overlying the posterior part of the ovary. The vulva opens to the exterior ventrally and slightly in front of the tail papilla. In front of

the vulva an indistinct oval structure is sometimes apparent. Its function is not known.

In the larvae the cuticle appears to undergo some change after being in contact with the blood of the host for some time and staining is then difficult. Mature females take up such stains as Nile Blue and glychaemalum much more readily.

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# A NOTE ON AN UNIDENTIFIED FUNGUS IN THE BODY CAVITY OF TWO THYSANOPTEROUS INSECTS

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(With 1 Figure in the Text)

A list of entomophagous fungi was published by Gray in 1858, and since then a great many more have been recorded. Accounts of those found in the body cavity of scale insects have been given by Schwarz (1924), and by Brues & Glaser (1921). These are apparently symbiotic and allied to *Saccharomyces*. Several species of *Coelomyces* have been found in the body cavity of mosquito larvae and described by Keilin (1921, 1927) and Iyengar (1935).

While the writer was working on the distribution of a nematode parasite of *Aptinothrips rufus* Gmelin she found, in the body cavity of this insect, spores of a fungus which appears to be different from any entomophagous species so far described. It was submitted to Dr Petch, who stated that identification was impossible until further stages could be obtained. So far attempts to culture it have been unsuccessful.

About 17,000 specimens of *A. rufus* were dissected and 35 were found to be attacked by the fungus. One hundred specimens of *Limothrips cerealium* Hal. were also examined, and in one of these were found spores (Fig. 1) identical with those from *Aptinothrips rufus*.

The spores are colourless and about 0·01 by 0·04–0·06 mm. in size. They are straight or slightly curved, and large terminal vacuoles are often present. Transverse fission appears to occur. I found no trace of a mycelium, and in no case were chains of spores seen.

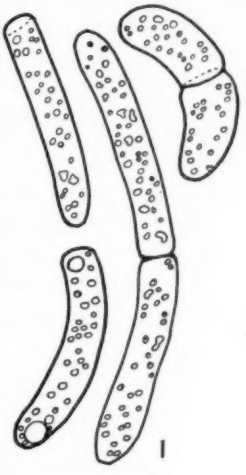

Fig. 1.

The presence of the fungus resulted in the sterility of the host except in one instance. In this exceptional case infection was very slight, only 20 or 30 spores being present. As a rule several hundreds were found, and occasionally they were so numerous that they almost filled the body cavity. Externally the infected thrips appeared healthy and normal though sometimes a little paler than their fellows.

Infection is most common in the spring and early summer. The specimens of *Limothrips cerealium* were collected from the wheat on Broadbalk field, Rothamsted, July 30, 1934. *Aptinothrips rufus* was taken at intervals from January 1933 to January 1935, from a number of grass plots in the classical field, Park Grass.

Twenty-nine of the 34 infected thrips from Park Grass were found on plots which have never been treated with lime, but statistically there was no correlation between the *p*H values of the soil and the distribution of the fungus.

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